Analytical Methods

An Integrated Approach to a Portable and Low-Cost Immunoassay for Resource-Poor Settings**

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The development of technology for use in resource-poor countries encounters a specific type of challenge not ordinarily faced in academic science: the technology must be inexpensive and it must work with minimal infrastructure. This challenge is particularly severe when the problems being solved are, by their nature, ones that require high-technology solutions. For these kinds of problems, the elegance of the solutions must lie in the use of science to guide the assembly of readily available components into a simple, workable, and well-integrated package. In this paper, we describe an integrated approach to a miniaturized immunoassay called a "POCKET immunoassay" ("POCKET" is short for *po*rtable and *c*ost-*e*ffec*t*ive). This immunoassay has, we believe, the potential to be inexpensive and operable with minimal equipment and technical skills, and shows an analytical performance approaching that of enzyme-linked immunosorbent assays (ELISA) performed in a bench-top format in clinical laboratories.

A top priority for improving health in developing countries is technology for simple, affordable diagnosis of infectious diseases.[1] Immunoassays such as ELISA are the most reliable and widely used methods for detecting antigens and antibodies, but they require expensive and bulky instruments for optical detection, hours of incubation for diffusion-limited reactions on the surface, and many steps of pipetting.[2,3] These constraints prevent the use of ELISA in settings that require low-cost or compact equipment, and in environments that lack electricity or trained personnel. One application with these requirements is the detection of infectious diseases in the field in developing countries;[1,4,5] other potential uses include point-of-care diagnostics by first responders and in health clinics,[6] and detection of biological warfare agents in the field.[2,7] Immunochromatographic assays (also known as "strip tests" and "lateral-flow

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[**] This research was supported by DARPA/NSF (ECS-0004030) and NIH (GM 65364), and used the MRSEC shared facilities supported by the NSF under Award No. DMR-9809363. V.L. was a recipient of a postdoctoral fellowship from the Swiss National Science Foundation. A.S. was a recipient of a Howard Hughes Medical Institute Predoctoral Fellowship. We thank Tyler Aldredge of the Center for Genomics Research for technical assistance.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

  DOI: 10.1002/anie.200353016

assays") are simple to operate, rapid, and commercially available, but they are less sensitive than ELISA, and give primarily yes/no results;[8] current work focuses on improving their sensitivity and capacity for quantitative analysis.[8–10] Moreover, although they are less expensive than many tests, they are still too costly for widespread use in developing countries, and for applications that require high-throughput analysis such as screening of blood samples.[4] As such, an immunoassay that is portable, rapid and simple to operate (like immunochromatographic assays), and that offers parallel, quantitative analysis and a strong, reliable analytical performance (like bench-top ELISA), will be a useful tool of detection in settings for which neither strip tests nor conventional ELISA are appropriate.

We take a comprehensive approach to the design of the assay by miniaturizing and integrating both the immunoassay and the detection device. The immunoassay in this study is performed in an inexpensive, miniaturized platform (made by soft lithography) that is compatible with microfluidics. Microfluidic immunoassays offer several advantages relative to microwell plates, which include kinetically rapid reactions at the surface, and the potential for automated fluid delivery and analysis of many samples in parallel.[11–14] The use of ELISA in microfluidics, however, poses two problems: The generation by enzymes of freely diffusible products makes detection difficult under conditions of continuous flow, and the small cross-sectional path length in microchannels limits the sensitivity of assays using simple optical detection. We address both of these problems in our approach for the immunoassay (Figure 1): instead of enzyme-conjugated secondary antibodies, we add antibodies conjugated to 10 nm gold colloids, followed by a solution containing silver nitrate and hydroquinone (as reducing agent). The gold colloids catalyze the reduction of silver ions to silver atoms; in turn, the solid silver catalyzes the further reduction of silver ions.[15] The resultant silver film, whose opacity is a function of the concentration of the analyte, partially blocks the transmission of light through the transparent polystyrene plate. Because the opaque silver product is attached to the surface, reduction of silver may be an effective method of amplification for microfluidic devices that operate under continuous-flow conditions, and may overcome the limitation of a small path length for optical detection of molecules in microchannels.

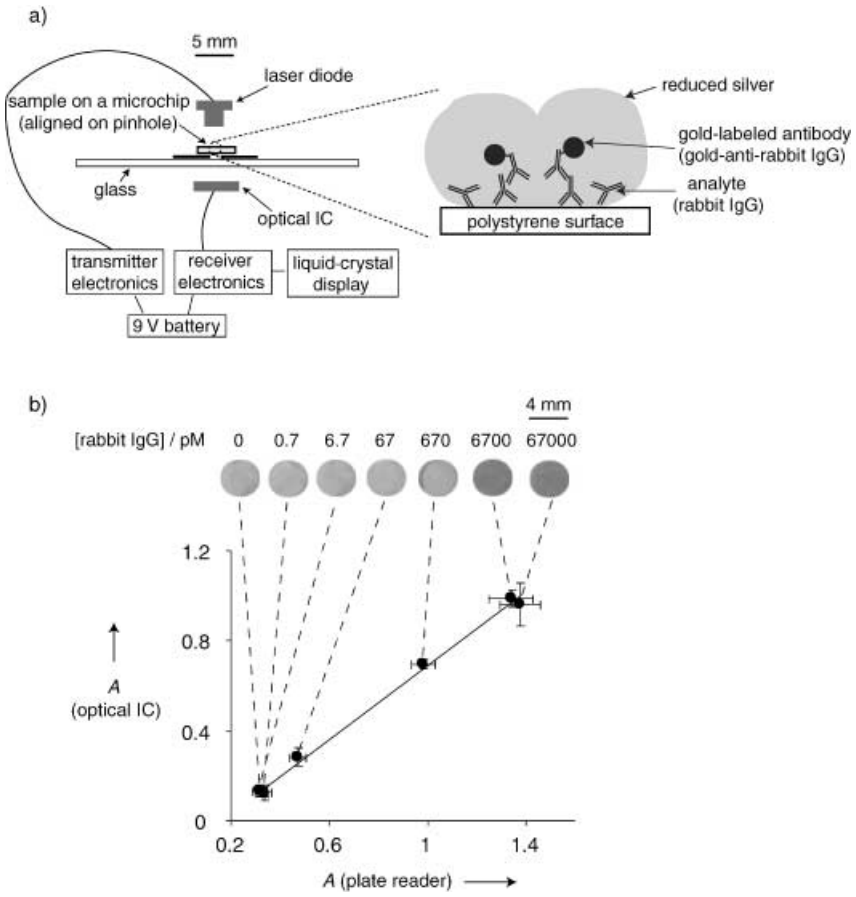

Figure 1. Schematic representation of the POCKET immunoassay, and performance of the optical detection device. a) Red light from the laser diode passed through the silver-coated microwell containing the sample to the optical IC. A pinhole was used to block stray light that did not pass through the sample. The laser diode and the optical IC were driven by the same circuit, which also had an integrated liquid-crystal display that showed the measured transmittance value; b) an immunoassay using silver reduction was performed on a 96-well plate that detected rabbit IgG. Optical micrographs of the silver films on microwells are shown for each sample. The apparent absorbance of each microwell was measured by an optical IC, and compared to its reading by a UV/Vis plate reader; both measurements were made at 654 nm. The best-fit line by linear regression has a correlation coefficient of 0.989, slope of 1.12, and intercepts the *y* axis at 0.16.

A detector for use in the field should be compact, low-cost, battery-powered, and if possible, reusable. Ideally, it should operate under different conditions in the field, such as direct sunlight. We designed and built a detector that satisfies these requirements, by measuring the transmission of light through the silver film. The detector consisted of an InGaAlP red semiconductor laser diode (654 nm) as the light source, and an optical integrated circuit (IC; which contains a photodiode, an amplifier, and a voltage regulator; it has a peak sensitivity wavelength of 700 nm) as the photodetector. To enhance the utility of the detector in direct sunlight, we used pulse modulation of the optical signal at 1 kHz to filter out noise from ambient light (most of which is at 0 Hz). This feature permitted measurements to be made under ambient light in the laboratory (as were all measurements shown in this report); neither shining a flashlight onto the detector nor bringing the device outdoors in daylight produced a change in the background signal. (We believe that, in the future, other types of modulation can be used to further increase the signal-to-noise ratio.) The entire detector was powered using a single 9 V battery (for over three hours of continuous usage), making it suitable for transportation and use in the field in conditions without ground electricity. We also connected the optical IC to a liquid-crystal display to obviate the need for a multimeter. The components for this reusable and portable detector were bought from commercial vendors for $45. (See

Supporting Information for the details of the circuit design.)[16]

We first characterized the performance of silver reduction with optical detection by the portable detector by performing an immunoassay of a model antigen in microwell plates. The concentration of rabbit IgG was determined in an immunoassay featuring a series of dilutions that spanned five orders of magnitude in concentrations (Figure 1b). The opacities of the samples were measured by the optical IC and by a bench-top UV/Vis absorbance plate reader. For comparison with the data from the plate reader, transmittance values reported by the optical IC were converted to apparent absorbance values (which accounted for both absorption and reflection of the incoming light by the silver film). The optical IC produced readings in excellent agreement with those of the plate reader (correlation coefficient of 0.996). The imperfect agreement between the two measurement methods may have resulted from inhomogeneous silver deposition.[16]

We compared the analytical performance of our method of detection (silver reduction with the low-cost, portable detector) to that of ELISA using the most common reporting systems with bench-top plate readers: absorbance, fluorescence, and chemiluminescence (Figure 2a). For this comparison, we used ELISA substrates that are highly sensitive. The titration data were fitted to sigmoidal curves, the best-fit curves of all methods were normalized to between 0 and 1, and the sensitivities and limits of detection were calculated [16] For an immunoassay detecting rabbit IgG, the most sensitive to the least sensitive method (as defined by the slope of the titration curve in the middle of the linear range of detection, in normalized units per 100 pM) is: chemiluminescence (0.19), fluorescence (0.12), silver (0.08), and absorbance (0.04). For limits of detection, the assays with the lowest limit to the highest limit were: chemiluminescence (22 pM), absorbance (55 pM), silver (89 pM), and fluorescence (163 pM). Immunoassays using silver reduction showed an average overall standard deviation of 7% (for multiple independent measurements of the same concentration of analyte) when measured by the plate reader, and 13% when measured by the optical IC, compared to 9–15% for the ELISA measurements. Thus, the overall analytical performance (sensitivity, limit of detection, and reproducibility) of the POCKET immunoassay in the microwell format approaches that of bench-top ELISA.

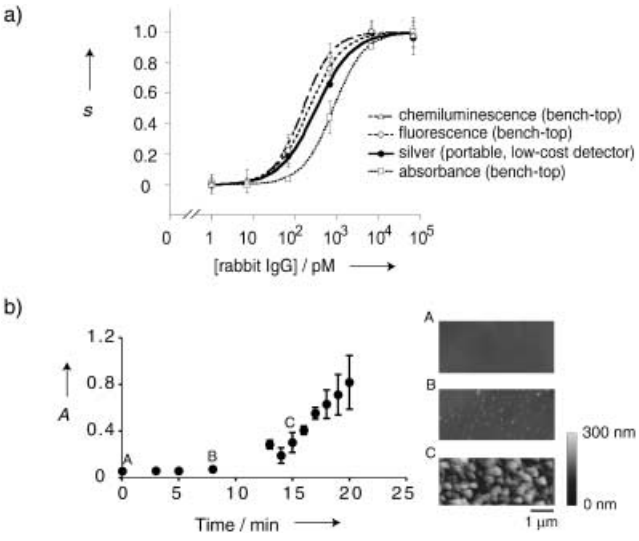

Figure 2. Performance of the POCKET immunoassay. a) Comparison of the POCKET immunoassay using detection by silver reduction and optical IC with detection by absorbance (pNPP substrate), fluorescence (AttoPhos substrate), and chemiluminescence (Supersignal ELISA Femto, a derivative of luminol, as substrate). Standard deviations of triplicates in a single experiment are shown as error bars (see Supporting Information for the procedure for normalizing the signal for different assays). In concentration units, 6.7 pM corresponds to 1 ng mL^{-1}. S = normalized signal; b) kinetics of silver deposition in an immunoassay (for all samples, the concentration of rabbit IgG was 67 nM, and the dilution of gold-labeled anti-rabbit IgG was 1:100). The microwells were incubated in silver enhancement solutions for the indicated times, the reaction was quenched with sodium thiosulfate, and the apparent absorbances of the silver films were measured by a UV/Vis plate reader. AFM images of samples at three different time points are shown. Standard deviations of triplicates in a single experiment are shown as error bars.

We characterized the process of silver deposition by performing an immunoassay for rabbit IgG in microwells, and stopping the silver reduction at various time points. As determined by UV/Vis spectroscopy, after incubation with the silver enhancement solution, silver reduction exhibited an initial slow-growth phase, and then proceeded rapidly at an approximately linear rate (Figure 2b). Analysis of the same samples by atomic force microscopy (AFM) confirmed that the increase in opacity of the surface correlated with the growth of silver particles (Figure 2b). In our assays, quenching the reaction after 10–20 min (see Supporting Information for specific times for each assay) resulted in reproducible amounts of silver that were formed for triplicates in a single experiment. A normalization procedure, such as the one described in Figure 2a (or other methods for running calibration curves) can be used to account for day-to-day fluctuations in the rate of silver deposition (due to an increase in temperature, for example, which increases the rate of silver deposition).

We developed the immunoassay in a microfluidic format by quantifying anti-HIV-1 antibodies in the sera of HIV-1-infected patients (Figure 3). The microfluidic device was fabricated in poly(dimethylsiloxane) (PDMS) using soft lithography.[17] For comparison, we also performed the same immunoassay in the microwell format. In both formats, the opacity of the silver film was measured using the portable detector. Incubation times required for each reagent were 10 min in the microfluidic device, and 1–3 h in the microwells (that is, a 6- to 18-fold reduction in the time required for this part of the assay). The POCKET immunoassay, in both the microwell and microfluidic format, can reliably distinguish the sera of HIV-1-infected patients from those of noninfected patients (Figure 3). Moreover, the assay in both formats can detect quantitative differences in the amount of anti-gp41 in the sample (Figure 3). At high concentrations of serum (low dilutions), the lower signals of the negative controls in the microfluidic device compared to microwells may have resulted from better washing of antibodies that were nonspecifically bound to the surface. We believe that in the

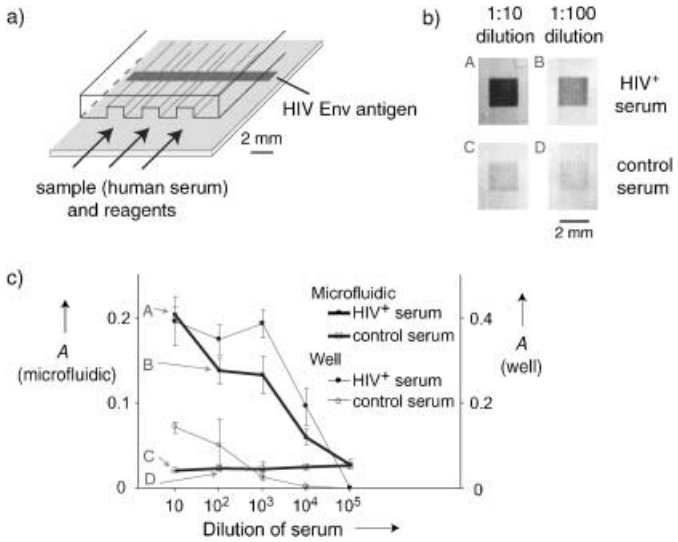

Figure 3. Detection of anti-HIV-1 antibodies in human-patient sera using the POCKET immunoassay. a) Schematic representation of a microfluidic device that detects anti-HIV-1 antibodies. The HIV Env antigen is patterned onto a polystyrene surface as a stripe, and a slab of PDMS with microchannels is placed orthogonally to the stripe. A sequence of reagents (blocking buffer, human serum sample, gold-labeled anti-human IgG, and the silver enhancement solution) is added to the microchannels using pressure-driven flow. Analysis of many dilutions was achieved in parallel by adding a different dilution of the human serum sample to each microchannel; b) photographs of the detection areas with reduced silver films; c) apparent absorbance values from an immunoassay detecting different dilutions of sera from HIV-positive (HIV^+) patients and control patients. For comparison, the assay was also performed in a 96-well plate. Standard deviations of triplicates in a single experiment are shown as error bars. The apparent absorbance values of the samples shown in b) are shown in this graph.

future, the pipetting steps of ELISA in microwells can be automated in the microfluidic device, although we have not implemented this feature yet in our device.

This study offers an alternative approach to other efforts for miniaturizing immunoassays for portable use. Methods for analysis of biomolecules on microchips include electrochemical detection,[12] electrical detection,[18] and integrated on-chip optical detection.[7,19,20] Compared to the detection of colorimetric and fluorescent substrates in solution, detection of silver reduction catalyzed on gold colloids (a method that has been used in other applications to analyze proteins[15,19,21,22] and DNA[23–26]) offers a number of advantages: 1) It is an effective method of signal amplification under conditions of continuous flow in microfluidics; 2) it circumvents the problem of a small path length in microchannels; 3) silver film, unlike fluorescent substrates, does not photobleach; 4) silver, unlike solutions of optically active molecules, is stable for months, which allows results of immunoassays to be kept for long-term use (after 23 days of storage in ambient laboratory conditions, the absorbance readings changed by 1.5%); 5) silver films block light at a broad spectrum of wavelengths (the absorbance of silver showed a maximum variation of 20% from 400 to 1000 nm), whereas chromophores and fluorophores are active only at specific wavelengths. With silver reduction, a wide variety of laser diodes and photodetectors (of any wavelength) can be used in the detection device.

Overall, the integrated POCKET immunoassay has several advantages: 1) It is low-cost and portable, and therefore is appropriate for use in the field; 2) the reporter method of silver reduction is compatible with the use of microfluidics under continuous flow conditions, which decreases the time required for the assay and makes possible a simplified delivery of reagents; 3) the optical detector is battery-powered, reusable, and, with pulse modulation, operable under field conditions such as direct sunlight; 4) the analytical performance of this integrated miniaturized device approaches that of ELISA using relatively expensive benchtop equipment. The POCKET immunoassay may therefore be appropriate for applications in resource-poor settings, including the diagnosis of infectious diseases in developing countries.

Received: October 6, 2003 [Z53016]

Keywords: analytical methods · colloids · immunoassays · microfluidics · proteins

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