A MULTI-STEP IMMUNOASSAY USING DRY, PATTERNED REAGENTS IN A TWO-DIMENSIONAL PAPER NETWORK FORMAT

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ABSTRACT

Here we demonstrate a multistep immunoassay using the controlled release of reagents patterned on a single porous membrane. Reagents are patterned, dried, and stored on-device for later rehydration, which removes the need for a cold chain and reduces user steps through the on-strip combination of signal enhancement reagents. The limit of detection for the malaria antigen *Plasmodium falciparum* histidine-rich protein 2 (*Pf*HRP2) achieved using these devices is comparable to ELISA. This work demonstrates the viability of patterning and drying reagents directly onto a paper device for dry reagent storage and subsequent controlled release in paper-based assays.

KEYWORDS

Multistep immunoassay, controlled release of dry reagents, paper microfluidics, dry reagent storage

INTRODUCTION

Lateral flow tests (LFTs) have been widely accepted for a variety of applications, ranging from home pregnancy tests to rapid diagnostic tests for infectious diseases in low-resource settings. LFTs have great appeal because they are low-cost, rapid, easy to use, and require little to no instrumentation to interpret results. More recently, 2D and 3D paper-based devices have been demonstrated with capabilities extending far beyond those offered by traditional LFTs. One characteristic of most of these newer paper-based devices that remains consistent with early LFTs is the inclusion of dried reagents in the assay device. This approach reduces user steps, removes the need for a cold chain, and facilitates device automation, thus improving the robustness, affordability and ease-of-use of the device while decreasing the equipment needs. These four key traits are crucial for designing devices appropriate for point-of-care diagnosis in low-resource settings [1, 2].

Traditionally, conjugate pads have been used to store dry reagents in both lateral flow [3, 4] and conventional microfluidic devices [5]. However, there are two significant disadvantages to using these separate pads: they offer limited control over the release of rehydrated reagents, and they require additional materials and components that add to manufacturing costs. Other methods for dry reagent storage in microfluidic devices have achieved such storage directly within the channels of these devices, for later controlled release. Some examples of these techniques include cavities in channel walls to control the reconstitution of dried proteins [6], and "reagent integrators" to store and subsequently release predetermined dilutions of reagents into microfluidic devices [7]. Our earlier work sought to develop analogous methods for controlled release of reagents stored directly on paper microfluidic devices [8]. To achieve those goals, we created novel methods for printing reagents on porous substrates to enable controlled spatial and temporal concentration gradients of reagents rehydrating during capillary flow within a porous device.

Here we present an application of those methods in the implementation of a signal-enhanced immunoassay for the malaria antigen *Plasmodium falciparum* histidine-rich protein 2 (*Pf*HRP2), using reagents patterned and stored on a single porous membrane. This work differs from previous demonstrations by our group [4, 9] because all assay reagents were patterned and stored dry directly on the nitrocellulose membrane, a significant simplification of conventional methods that require storage in separate pads and subsequent placement in the device. Another method for the storage and rehydration of reagents directly from porous devices has been described by Abe *et al.* [10]. The work described here, however, enables controlled release of rehydrating reagents, as well as mid-flow combination of assay reagents.

EXPERIMENTAL

Untreated nitrocellulose membranes (Millipore Hi Flow Plus 135, Millipore, Billerica, MA) were cut into 3-inlet networks using a CO_2 laser (Universal Laser Systems, Scottsdale, AZ). After cutting, the first inlet was blocked with 14 µl of an aqueous solution containing 0.125% poly(vinyl pyrrolidone) + 0.125% bovine serum albumin + 2.5% sucrose + 7.5 mM sodium azide + 0.1% Tween-20, then placed in a desiccated oven at 37°C for 2 hours. The antigen-capture line (0.375 µl, 1 mg/ml mouse monoclonal anti-*Pf*HRP2 IgM, Immunology Consultants Lab), and a process control (0.375 µl, 0.5 mg/ml ImmunoPure Antibody goat-anti-mouse IgG, Thermo Scientific) were immobilized at the downstream end of the common channel of the device. The detection antibody (Immunogold conjugate mouse monoclonal anti-*Pf*HRP2, BBInternational) was patterned for rehydration on the first inlet of the device. GoldEnhance LM gold enhancement solution (Nanoprobes, Yaphank, NY) was used for enhancement; 2 µl of each "enhancer" solution, "activator" solution, and "initiator" solution were printed on the third inlet. All of the reagents were patterned using a piezoelectric spotter (SciFLEXARRAYER S3, Scienion AG) (Figure 1). After printing, strips were wrapped in foil to protect them from light, then dried in a desiccator overnight and stored until use.

Nitrocellulose devices were affixed to a PMMA substrate using double-sided tape (Scotch®, 3M, St. Paul, MN). However, no cellulose wicking pad was used. An untreated glass fiber pad (Ahlstrom, Helsinki, Finland), cut using the CO_2 laser cutter, was placed at the upstream end of each inlet as a fluid application zone (Figure 1). A 30 µl "mock

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sample" consisting of *Pf*HRP2 spiked into fetal bovine serum (FBS, Certified, One Shot™, US Origin, Gibco ®, 16000-077, Invitrogen, Carlsbad, CA) was applied to the first inlet, while 40 µl and 100 µl phosphate buffered saline (PBS) were applied to the second and third inlets, respectively. The mock sample rehydrated the gold conjugate antibody in the first inlet, and PBS rehydrated and combined the gold enhancement reagents in the third inlet. Rehydrated reagents were then delivered sequentially to the detection region of the assay. Time-lapse uncompressed .avi videos of assay experiments were acquired using a web camera (Logitech, Fremont, CA) at 1 frame per 30 seconds for 1 hour (Figure 2).

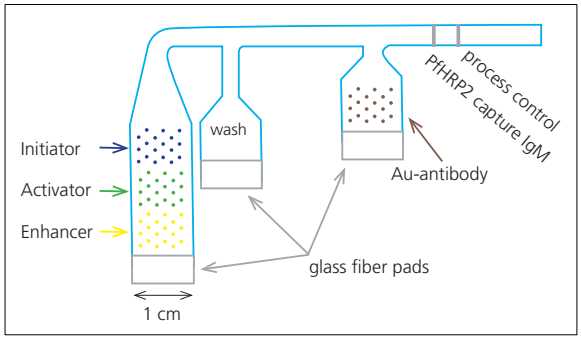

Figure 1: Schematic of patterned 2D paper network PfHRP2 assay indicating locations of patterned reagents.

Uncompressed .avi files were analyzed with ImageJ to determine the signal development over time (Figure 3). The reported intensity values were determined as follows: average grayscale intensity of the test line was quantified for each frame, and background intensity was subtracted and then normalized by the background to account for lighting variations. The enhancement ratio was determined by quantifying the fully enhanced assay signal at 60 minutes, and dividing it by the unenhanced signal at 15 minutes. To quantify signal for varying concentrations of *Pf*HRP2 (Figure 4), strips were scanned to uncompressed image files with a flatbed scanner (Epson Perfection V700 Photo, Epson, Long Beach, CA) with gamma set to 1. Scanned images were quantified using Matlab (MathWorks, Natick, MA). The grayscale intensity of the test line was measured, and then the background value was subtracted. The limit of detection (LOD) of the assay was calculated as follows: $LOD = (LOB+1.645\sigma_t)/m$, where LOB = limit of blank (to be explained below), σ_t = the standard deviation of the lowest antigen test, and m is the slope of the signal response curve between zero and the lowest antigen test. The limit of blank is defined as: $LOB = average_{no\ antigen} + \sigma_b$, where σ_b = standard deviation of the blank [11].

RESULTS AND DISCUSSION

The multi-component gold enhancement system that we used for signal amplification loses functionality if the components are mixed prior to drying (data not shown). Thus, components must be stored dry in separate regions and recombined on the device for use in the assay. Previous work has shown effective rehydration and combination of these gold enhancement components from storage on multiple glass fiber pads in the context of a malaria assay [4]. Here, we observed that gold enhancement reagents printed separately on one device leg combine upon rehydration to yield a bolus of complete gold enhancement solution, which is then capable of enhancing the gold signal generated in a PfHRP2 assay (Figure 2).

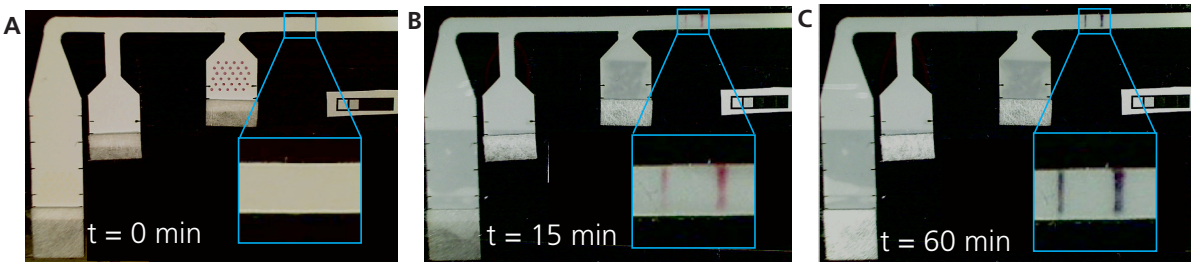

Figure 2: Time series of images illustrating gold signal development and enhancement. A) Device prior to fluid addition. 30 µl of a mock sample consisting of PfHRP2 spiked at 100 ng/ml into FBS was applied to the first inlet. Immediately afterward, 40 µl and 100 µl PBS were applied to the second and third inlets, respectively. B) The initial gold signal appears 15 minutes after fluid addition. C) After 60 minutes, the gold enhancement has increased the signal to 3.2 (s.d. 0.2) times the unenhanced intensity.

After 60 minutes, the rehydration and mid-flow combination of printed gold enhancement reagents produced a 3.2-fold signal enhancement (n=4, s.d. 0.2), relative to the initial signal observed at 15 minutes (Figure 3). Just 2 µl of gold-antibody conjugate, and 6 µl of total gold enhancement reagents were used. These volumes are significantly lower than those required in previous demonstrations of signal enhancement in paper-based assays [4, 12]. Though the enhancement ratio achieved with this particular gold enhancement reagent is modest, this is a clear demonstration of the viability of patterning and drying reagents for dry reagent storage in paper-based assays. Furthermore, quantifying the assay signal confirmed reproducible signal at concentrations as low as 5 ng/ml, with very low background signal in the negative controls (Figure 4). Using this data, the analytical LOD was determined to be 3.6 ng/ml, which is comparable to the reported LOD for *Pf*HRP2 by ELISA of 4 ng/ml [13].

CONCLUSIONS

This model system is a simple and effective demonstration of the utility of multiple reagents patterned sequentially on a porous device and is just one example of the potential applications enabled by printing reagents directly on porous devices for controlled rehydration and use in assays.

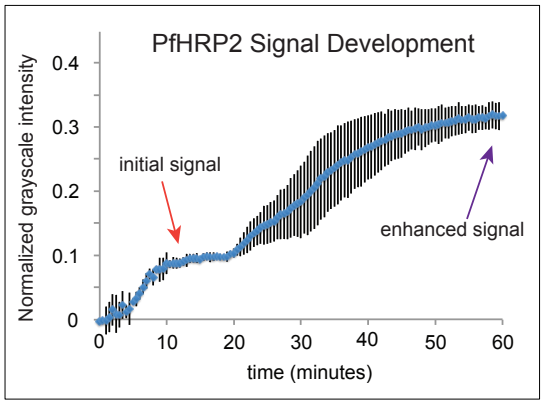

Figure 3: Assay signal vs. time. Time-lapse uncompressed avi files were acquired (1 frame per 30 sec.), and were analyzed using ImageJ (n=4, error bars = s.d.). The large error bars between 25 minutes and 50 minutes are due to variability in the time at which enhancement began, rather than the rate of enhancement

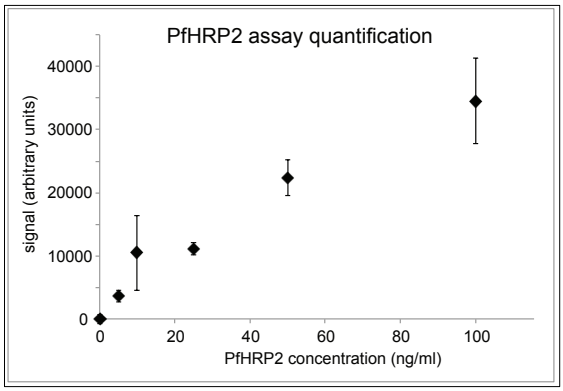

Figure 4: Assay results for varying concentrations of PfHRP2 antigen. Plot of the average signal for each concentration of antigen (n=4, error bars = s.d.). From this data, the LOD was determined to be 3.6 ng/ml.

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