Simultaneous Concentration and Detection of Biomarkers on Paper

Erik Jue, Allison Yip, Andrew Berg, Stephanie Wang, Alexandra Kivnik, Phuong Nguyen, and Dr. Daniel Kamei
Department of Bioengineering, University of California, Los Angeles, CA

Abstract

Malaria is a leading cause of death and disease in developing countries, leaving young children and pregnant women at highest risk for infection. In order to effectively treat malaria, a device capable of producing accurate results is essential for early detection of the disease. The lateral-flow immunoassay (LFA) is an inexpensive point-of-care (POC) paper-based diagnostic device with the potential to rapidly detect disease biomarkers, such as those characteristic of malaria, in resource-poor settings. Although LFA is inexpensive, easy to use, and requires no laboratory equipment, it is limited by its sensitivity, which remains inferior to that of gold standard laboratory-based assays. The Kamei Lab has previously utilized various aqueous two-phase systems (ATPSs) to enhance LFA detection. In those studies, the sample was first concentrated by an ATPS in a test tube and could only be applied to LFA after it had been extracted manually. Here, we bypass the extraction step by integrating a polyethylene glycol-potassium phosphate ATPS with downstream LFA detection in a simple, inexpensive, power-free, and portable all-in-one diagnostic device. We discovered a new phenomenon in which the target biomarkers simultaneously concentrate as the ATPS solution flows through the paper membranes. Our device features a novel 3-D paper well that was designed to exploit this phenomenon. Studies using this device, which were performed at room temperature in under 25 min, demonstrated a 10-fold improvement in the detection limit of a model protein, transferrin. Our next-generation LFA technology is rapid, affordable, easy-to-use, and can be applied to existing LFA products, thereby providing a new platform for revolutionizing the current state of disease diagnosis in resource-poor settings.

Introduction

An estimated 3.4 billion people, or almost half of the entire world population, live in regions that are prone to infection by malaria.[1] In 2012, there were an estimated 207 million total cases of malaria and approximately 627,000 deaths due to the disease, primarily affecting young children and pregnant women.[1] Of these deaths, 90% occurred in sub-Saharan Africa, where accurate diagnostic technologies are not readily available due to limited access to electricity, laboratory equipment, and trained personnel.[1,2] There is a critical need for earlier detection of the disease in order to enhance diagnosis, enable faster administration of treatments, and improve outbreak prevention.[3] To fulfill this unmet need, resource-poor countries require a next-generation point-of-care (POC) diagnostic assay that is accurate, rapid, equipment-free, simple to use, easy to interpret, and low in cost.[4]

Blood smears, the current gold-standard diagnostic laboratory assays for malaria detection, utilize microscopy to accurately identify malaria parasites directly from a drop of the patient's blood.[5] However, this procedure requires laboratory equipment, trained personnel, and extensive sample processing, which are all limited in resource-poor settings.[2] Therefore, a paper-based immunochromatographic device, which is easy to operate without laboratory equipment, is better suited for POC testing. Paper is advantageous for these settings because it is readily available, affordable, and provides an intrinsic wicking mechanism that can quickly transport fluids without the aid of an active pumping system. One common paper-based diagnostic device is the lateral-flow immunoassay (LFA), a rapid antibody-based test already used in many applications, including off-the-counter pregnancy tests and urine drug test kits. LFA can also be used for detecting biomarkers in saliva, which is advantageous in POC settings since sharps can be eliminated from the sample collection procedure. This leads to diagnostic assays that are simpler, more portable, and less costly than those that test with blood.[6]

However, LFA for the detection of *Plasmodium falciparum* lactate dehydrogenase (pLDH), an enzyme produced by the malaria-causing parasite *P.falciparum*, was shown to accurately diagnose malaria in only 78% of patient whole-saliva samples. In order to ensure more accurate results in saliva samples, the sensitivity of LFA must be improved, while maintaining its ease-of-use.

Many methods that improve LFA sensitivity alter the LFA device itself, but in doing so, often make the device less applicable for POC testing.[6] This is due to a combination of increased costs, increased assay times, reduced ease-of-use, required trained personnel, or required laboratory equipment. Rather than modify the LFA assay with a process such as signal amplification, the Kamei Lab previously focused on the pre-concentration of the target sample using ATPS.[7-10] The introduction of ATPS technology to the LFA system also has similar drawbacks such as increased assay times and reduced ease of use. However, in this project, our solution is able to reduce these incompatibilities with POC testing through the complete integration of the ATPS and LFA into a single packaged device.

Materials & Methods

Determining the Polymer-Salt ATPS Solution Volume Ratios

Polyethylene glycol 8000 (PEG, VWR) and potassium phosphate salt (5:1 dibasic to monobasic ratio, Sigma-Aldrich) were dissolved in Dulbecco's phosphate-buffered saline (PBS; Invitrogen). The equilibrium volume ratios (volume of the top phase divided by the volume of the bottom phase) were obtained by varying the w/w compositions of PEG and salt along the same tie line. The 1:1 and 9:1 volume ratio ATPSs were found and used for further experiments.

Preparation of Antibody-Decorated Dextran-Coated Gold Nanoprobes (DGNPs)

Dextran-coated gold nanoparticles were synthesized according to Min and coworkers with slight modifications.[11] To form functionalized DGNPs, the pH of the dextran-coated gold nanoparticle solution was adjusted to 9.0 using 1.5 M NaOH. For every 1 mL of dextran-coated gold nanoparticle solution, 8 µg of anti-Tf antibodies (Bethyl Laboratories) were added. The reaction mixture was placed on a shaker for 30 min to facilitate the formation of dative bonds between the antibodies and the dextran-coated gold nanoparticles. Free antibodies were removed by centrifugation. The pellet was resuspended in 100 µL of 0.1 M sodium borate buffer at pH 9.0.

Visualization of ATPS

In order to visualize the two phases of the ATPS, unconjugated dextran-coated gold nanoparticles, which are purple due to surface plasmon resonance,[12,13] and Brilliant Blue FCF dye (The Kroger Co.) were added to the 1:1 and 9:1 volume ratio PEG-salt ATPSs to form final solutions of 3 g. These solutions were well-mixed through vortexing and incubated at 25 °C. Pictures of the solutions were taken when the ATPS reached equilibrium.

The two phases of the ATPS were then visualized as they flowed along a paper membrane. Two 8 x 30 mm strips of fiberglass paper were laser cut with a VersaLASER 3.50 (Universal Laser Systems, Scottsdale, AZ). Subsequently, 50 mg of the mixed ATPS (corresponding to the 1:1 or 9:1 equilibrium volume ratio) containing Brilliant Blue FCF dye and dextran-coated gold nanoparticles were added to one end of the strips. Images of the resulting flow were captured at 0, 30, 105, and 300 sec.

To visualize the phase separation of the ATPS within the 3-D paper well, 140 mg of a mixed ATPS containing Brilliant Blue FCF dye and dextran-coated gold nanoparticles were added to the paper well. The 3-D paper well was formed by stacking nine 8 x 10 mm laser-cut strips of fiberglass paper on one edge of an 8 x 60 mm laser-cut strip of fiberglass. After the mixed ATPS was applied to the 3-D paper well, 50 µL of running buffer (0.2% bovine serum albumin (BSA), 0.3% Tween20, 0.1 M Trizma base, pH 8) were added to the 3-D paper well. A

running buffer was added to assist the flow of the sample from the paper well to the rest of the device. Images were captured at 0 and 30 sec, at the addition of running buffer, and after completion of flow.

Detection of Tf with the 3-D Paper Well

LFA test strips utilizing the competition assay format were assembled similar to our previous studies, but with slight modifications. Specifically, the cellulose sample pad was replaced with a 5 x 20 mm fiberglass paper, which connected to a nitrocellulose membrane containing the test and control lines. At the beginning of the sample pad, a 3-D paper well composed of multiple strips of fiberglass paper was used. For experiments using the 1:1 volume ratio ATPS, the well was composed of five (four 5 x 7 mm strips plus the bottom sample pad) layers of fiberglass paper. To start the test, 40 µL of the mixed 1:1 volume ratio ATPS containing DGNPs and a known concentration of Tf were added to the paper well, followed by the addition of 50 µL of running buffer. Images were captured after 10 min. For experiments using the 9:1 volume ratio ATPS, 20 layers of paper (nineteen 5 x 7 mm strips plus the bottom sample pad) were used to form the paper well. 200 µL of the mixed 9:1 volume ratio ATPS containing DGNPs and a known concentration of Tf were added to the paper well, and allowed to incubate for 10 min, followed by the addition of 100 µL of running buffer. After another 10 min, images were captured by a Canon EOS 1000D camera in a controlled lighting environment.

Results & Discussion

Overview

We have developed a paper-based POC diagnostic that fully integrates an aqueous two-phase system (ATPS) with LFA. ATPSs are liquid-liquid extraction systems that are capable of concentrating the target biomarker into just one of the two phases. These systems are also compatible with POC diagnostic devices, such as LFA, since their size can be varied to accommodate individual samples. Furthermore, because both phases are primarily composed of water, they provide a mild environment for biomarkers in the system. Previous experiments by the Kamei Lab have demonstrated that the biomarker-containing phase of a polyethylene-glycol and potassium phosphate (PEG-salt) ATPS can be extracted with a syringe kit and applied to LFA for enhanced biomarker detection and sensitivity.[9,10] Our new design bypasses this extraction step by allowing for the direct addition of a mixed phase ATPS solution onto the device. The biomarker-containing phase automatically separates to the leading front of flow and is processed downstream by LFA. In removing this extraction step, trained personnel and a syringe kit are no longer required to operate the device.

Device Design and Innovation

Our device takes advantage of a newly discovered phenomenon in which the ATPS solution separates as it flows towards the LFA detection zone, allowing for the concentration and detection steps to occur simultaneously within the paper (Figure 1). Although the precise mechanism is still under investigation, our studies suggest that the paper network speeds up ATPS phase separation by interacting with the various ATPS components and promoting coalescence of similar phase domains. To further capitalize on this phenomenon, we expanded the paper device vertically, thereby increasing the cross-sectional area of flow and exploiting the effects of gravity on macroscopic separation. This 3-D paper component also has the ability to process larger and more dilute volumes of sample, leading to greater concentration-fold improvements.

Our 3-D paper diagnostic is the first to demonstrate ATPS phase separation in paper and successfully yields a 10-fold improvement in the detection limit of a model protein, transferrin, which is used to mimic the size and hydrophobicity of a protein biomarker. This

implementation improves the sensitivity of conventional LFA while maintaining its ease-of-use and time-to-result, and has the potential to rapidly identify pathogens that were previously undetectable with conventional LFA. We envision that, once fully developed, this platform technology could revolutionize the state of health care in resource-poor settings by providing rapid, accurate, and inexpensive diagnostics, leading to improved patient management, treatment, and outbreak prevention.

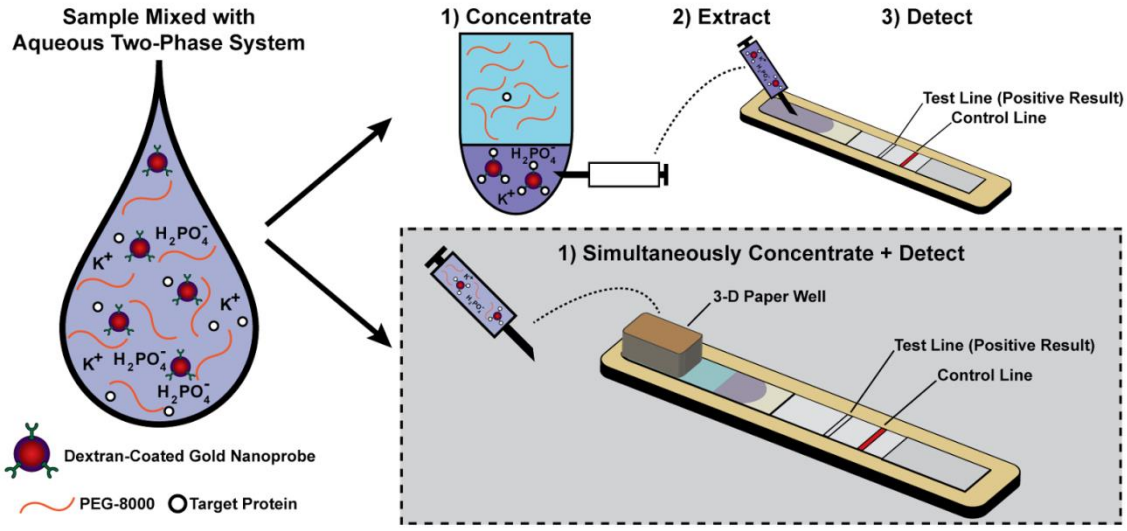

Figure 1: Unlike previous proof-of-concept studies performed by the Kamei Lab, which required ATPS separation in a test tube followed by extraction and application to LFA, our improved device simultaneously concentrates and detects biomarkers on paper (gray box).

Visualization of ATPS

The functionality of our device was first validated using a blue, hydrophilic dye and purple, more hydrophobic dextran-coated gold nanoparticles. Biomolecule partitioning in the ATPS is primarily dictated by relative hydrophilicity (since biomolecules tend to prefer the phase in which they experience the greatest attractive interactions) and size (since large biomolecules typically do not remain in the PEG-rich phase due to the presence of steric excluded-volume repulsive interactions with a greater number of PEG molecules). Accordingly, the dye partitioned extremely to the PEG-rich phase on top, whereas the dextran-coated gold nanoparticles partitioned extremely into the salt-rich phase on bottom. This allowed for visualization of the two phases of the PEG-salt ATPS and, since the nanoparticles can be functionalized with antibodies to fish for biomolecules in solution, also suggested the potential ability to predictively concentrate biomolecules in the bottom salt-rich phase (Figure 2).

The direct addition of a mixed ATPS to nitrocellulose paper also allowed for the visualization of ATPS phase separation. A mixed 1:1 volume ratio ATPS phase separated almost immediately within the paper (Figure 3), but the same did not occur for a mixed ATPS with a more extreme 9:1 volume ratio. Therefore, we designed a 3-D paper well to enable gravitational forces, which normally drive phase separation in test tubes, to also aid in enhancing this newly discovered phase separation phenomenon within paper. This, combined with the fact that the 3-D paper well increases the cross-sectional area normal to the direction of flow, allowed for the visualization of ATPSs with more extreme volume ratios (Figure 4). More extreme volume ratios result in a smaller, more concentrated leading front, and this is necessary because the LFA detection zone can only process a limited volume before it becomes fully saturated and non-functional.

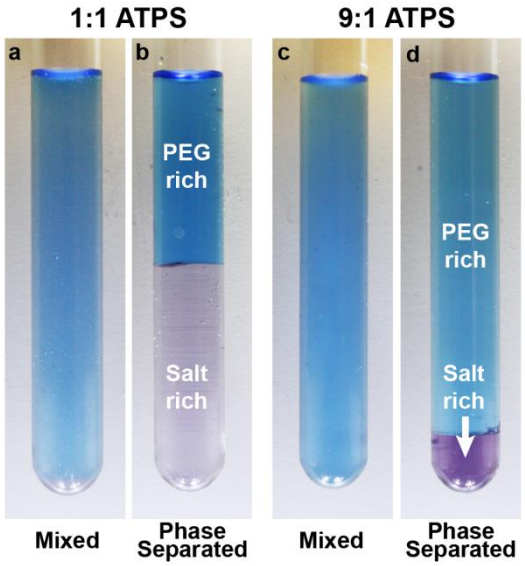

Figure 2: Brilliant Blue FCF dye and dextran-coated gold nanoparticles partitions extremely to the upper PEG-rich phase and lower salt-rich phases of the ATPS, respectively. (a) A mixed 1:1 volume ratio ATPS phase separated to form (b) two equal volume phases. (c) A mixed 9:1 volume ratio ATPS phase separated into (d) a larger PEG-rich phase and smaller salt-rich phase. The darker purple color of the 9:1 volume ratio ATPS indicates that the gold nanoparticle concentration in the bottom salt-rich phase has significantly increased.

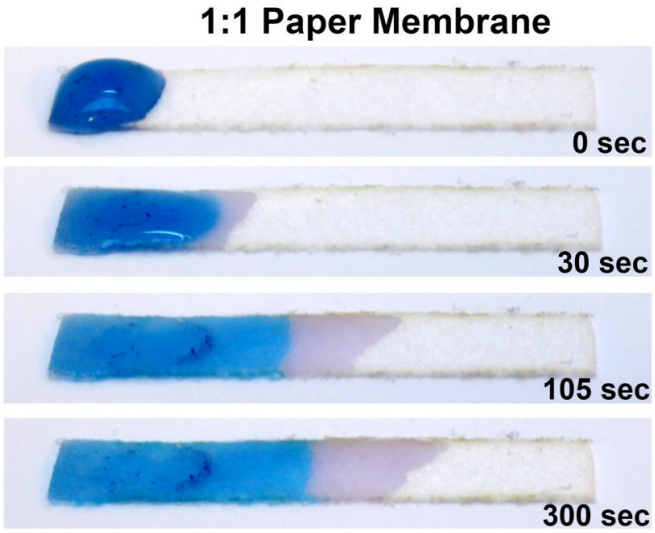

Figure 3: A mixed 1:1 volume ratio PEG-salt ATPS is added directly onto paper. The salt-rich phase (purple dye) separates more rapidly from the PEG-rich phase (blue dye) in paper than in the test tube.

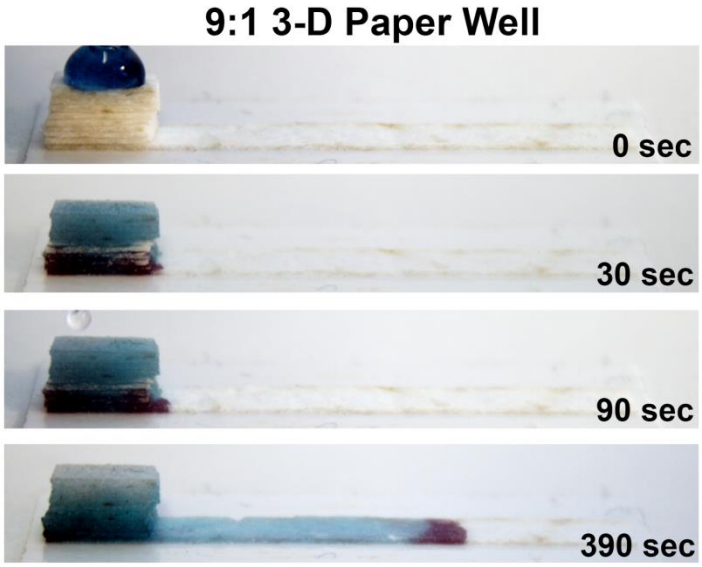

Figure 4: The paper well improves the phase separation phenomenon and enables use of a 9:1 volume ratio ATPS with greater concentrating effects. The darker purple front contains concentrated biomarkers and, if the paper well were integrated with downstream LFA, would encounter the detection zone before the remainder of the ATPS.

Detection of Tf with the 3-D Paper Well

The 3-D paper well technology was integrated with downstream LFA detection to create an all-in-one device with improved sensitivity over traditional LFA. As the 9:1 mixed ATPS was already shown to efficiently phase separate within our device (Figure 4), the Brilliant Blue FCF dye was no longer required. Instead, here we directly applied 9:1 mixed ATPSs containing anti-transferrin DGNPs and varying concentrations of the model protein transferrin to our combined ATPS/LFA paper-based diagnostic device. The DGNPs probe the solution and capture the transferrin model protein, and are then concentrated to the small, leading front of flow for downstream processing via LFA. By comparing samples applied to conventional LFA versus our device, we demonstrate a 10-fold improvement in the detection limit, or the lowest biomarker concentration resulting in a true positive result (Figure 5).

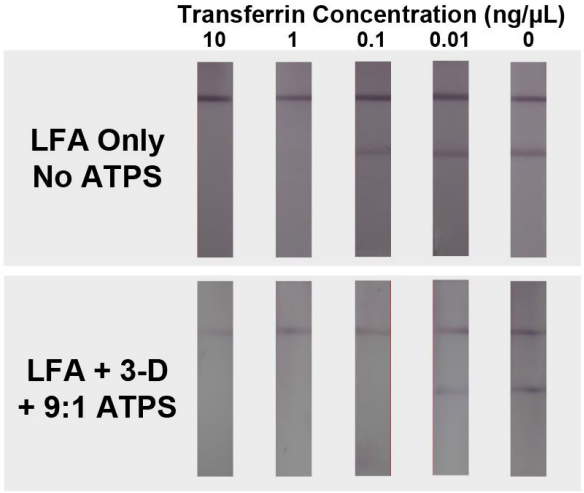

Figure 5: Traditional LFA is compared to our combined ATPS/LFA paper-based diagnostic device. In the competition assay, negative results are indicated by two bands, whereas positive results are indicated by one band. The detection limit of a model protein transferrin is improved 10-fold, from 1 ng/mL to 0.1 ng/mL. By lowering the detection limit, this technology has the potential to vastly improve disease diagnosis in resource-poor settings and transform the current state of healthcare.

Conclusion

Here we have demonstrated that our 3-D paper-based diagnostic device allows for the direct addition of an ATPS and results in a 10-fold improvement in the detection limit of a model protein Tf. A mixed ATPS applied directly to the paper membrane rapidly phase separates as it flows through the device, simultaneously allowing biomarker concentration and downstream detection with a reduced time-to-result. Although the precise mechanism for enhanced ATPS phase separation in paper still requires further investigation, we have shown that the paper speeds up the ATPS phase separation behavior and that 3-D paper architectures can further enhance this phenomenon. Expanding into the vertical dimension with the paper well enables processing of larger sample volumes and allows gravitational forces to aid in the phase separation, while the multiple layers of fiberglass membrane provide a greater cross-sectional area through which the domains of the ATPS can interact. This implementation improves the sensitivity of conventional LFA devices while maintaining ease-of-use and time-to-result. With 10-fold improvements in detection limit, our device has the potential to rapidly identify pathogens at lower concentrations and detect for malaria, which was previously limited by conventional LFA. This robust and portable device requires no electricity or sophisticated laboratory equipment and is ideal for POC applications in resource poor settings. The 3-D paper architecture can also easily be applied to existing commercial LFA products, transforming them to next-generation rapid diagnostics without inheriting the poor sensitivity characteristic of conventional LFA. In the future, our device can be modified to detect for the pLDH biomarker produced by malaria-causing parasites. Once fully developed, this platform technology has the potential to revolutionize the state of health care in resource-poor settings by providing rapid, accurate, and inexpensive diagnostics, leading to improved patient management, treatment, and outbreak prevention.

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