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Paper-based Vertical Flow Immunoassay (VFI) for detection of bio-threat pathogens

Peng Chen[a], Marcellene Gates-Hollingsworth[b], Sujata Pandit[b], Anson Park[c], Douglas Montgomery[c], David AuCoin[b], Jian Gu[a,*], Frederic Zenhausern[a,*]

[a] Center for Applied NanoBioscience & Medicine, College of Medicine – Phoenix, University of Arizona, Phoenix, AZ, USA
[b] Department of Microbiology and Immunology, University of Nevada School of Medicine, Reno, NV, USA
[c] School of Computing, Informatics and Decision Systems Engineering, Arizona State University, Tempe, AZ, USA

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ABSTRACT

Currently, the standard method for identifying biological agents of potential threats to national security and public health, such as pathogens, virus, and toxins, mainly rely on microbiological cultivation. This method is time-consuming and it requires sophisticated equipment and well-trained personnel, which are often unavailable in remote areas or at point-of-need. Therefore, an alternative rapid, simple, and sensitive method for detecting bio-threat agents is in crucial need. We report a paper-based Vertical Flow Immunoassay (VFI) device that can overcome these limitations. The VFI device utilizes a nanoporous nitrocellulose membrane encapsulated in a stainless steel filter holder. As the sample is pushed through the membrane, which is pre-functionalized with capture antibody, a sandwich assay is formed and colorimetric signal is generated to reflect the presence of target antigens. Through theoretical analyses of antigen-antibody binding process inside a porous membrane, we identified two critical factors – membrane pore size and sample flow rate that can be optimized to improve the assay sensitivity. Then, the effects were demonstrated through experimental studies using *Burkholderia pseudomallei* (the causative agent of melioidosis) as a model pathogen. The *B. pseudomallei* VFI was based on an immunoassay targeting the *B. pseudomallei* surface capsular polysaccharide (CPS). The experimental results agreed well with the theory showing that increasing the flow speed (up to 1.06 mm/s) and reducing the membrane pore size (down to 0.1 μm) could improve the sensitivity by at least 5 times. The VFI's limit-of-detection for CPS spiked in buffer solution was determined to be 0.02 ng/mL. The developed VFI shows great potential as a point-of-care tool for detection of bio-threat agents in a variety of clinical and resource-restricted conditions.

1. Introduction

During the last century, in parallel to progress made in basic and applied microbiology, there have been concerning advances made toward the weaponization of biological agents and biotoxins [1]. Currently, there are nearly seventy biological agents listed by the U.S. Department of Health and Human Services as potential threats to public health and national security. Exposure to these agents can cause a variety of human and animal diseases. *Burkholderia pseudomallei*, a soil and water dwelling bacterium that causes the deadly disease melioidosis in human and animals, is considered a high priority biological threat because of its potential to be weaponized and ability to produce fatal diseases. Recently, the total number of melioidosis cases worldwide has been estimated to be 165,000 of which 89,000 people die [2]. Infections vary from mild disease to overwhelming septicaemia within 24–48 h after symptom onset, and untreated cases of melioidosis have an even higher fatality rate of 90% [3]. Early diagnosis and treatment are key factors in improving disease management and lowering the mortality rate of infected patients [4].

Despite several advances in immunological and molecular approaches to detect *B. pseudomallei* [5], culturing of *B. pseudomallei* from clinical samples (e.g. blood, urine, pus, sputum, etc.) remains the "gold-standard" for melioidosis diagnosis. However, laboratory culture is very time-consuming and often challenging due to the low levels of bacteria present in many clinical samples. Culture and molecular diagnostic approaches (i.e. PCR) all require sophisticated equipment and highly-skilled personnel that are often unavailable in remote and resource-restricted regions. These difficulties can lead to the infection being misdiagnosed and grossly underreported in these areas. Moreover, recent statistical studies have found that the culture method is not a

* Corresponding author.
E-mail addresses: pengchen@email.arizona.edu (P. Chen), jgu10@email.arizona.edu (J. Gu), fzenhaus@email.arizona.edu (F. Zenhausern).

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reliable gold standard for diagnosis of melioidosis infection due to its low sensitivity [6]. Therefore, a simple, rapid, point-of-care (POC) diagnostic tool that can quickly and accurately detect bio-threat agents such as *B. pseudomallei* from clinical samples is highly desirable.

With the advancement of nanotechnology, paper materials have been utilized for biomedical applications [7]. A majority of the reported paper devices are designed in a lateral flow immunoassay (LFI) format where the fluid flow is parallel to the surface of the paper. This format typically consists of several segments (including a key nitrocellulose sensing paper membrane) joined together with a backing layer [8], and the testing fluid is transported through these segments by the capillary force. The main benefits of LFI include low cost, rapid results, flexibility and ease of use. However, in order to achieve an adequate flow rate, the pore size of the paper materials is limited to several micrometres and above, which could hinder the biomolecule capturing and thus the assay sensitivity. In addition, sample volume constraints and the difficulties of multiplexing are other hurdles that confront LFI development. Several LFI prototypes for multiplexing detection have been reported using paper device with branched geometry [9] and microarray [10]. Overall, the assay multiplexing is still limited and they require careful design of the membrane geometry to prevent cross-contamination so that the upstream reactions do not affect the downstream reactions.

One approach to improving upon the LFI platform has been the development of flow-through devices. This alternative format is similar to LFI in that it is a membrane based immunoassay, however, fluids are applied vertically to the surface of the membrane rather than parallel [11]. In recent years, there have been several reports on various designs and developments of vertical flow assays. One design was to simply replace the conventional lateral flow segments in a stacking manner, and let the liquid diffuse from the bottom to the top layers [12]. Another method was to push the reagents through one single membrane in steps, and allow for the targets to react with the reagents on the membrane. A vertical flow allergen microarray assay was built with more than a thousand binding sites to detect IgE activities in human serum samples with allergen components [13,14]. Similarly, an immunofiltration assay for simultaneously detecting of HIV p24 and hepatitis B virus antigens was recently reported [15]. Recent efforts have been made to investigate the possibilities of coupling the vertical flow assay with surface-enhanced Raman spectroscopy (SERS) toward higher sensitivity [16,17].

A LFI prototype was developed previously to detect *B. pseudomallei* using its surface capsular polysaccharide as the biomarker, but its sensitivity was not sufficient for early stage infection when the biomarker's presence was low [18,19]. In this study, the vertical flow format was investigated to develop a device for detecting bio-threat pathogens with improved sensitivity and better multiplex capabilities. Firstly, we built a theoretical model to simulate the bio-molecule binding process inside porous material, which led us to identify two critical factors – membrane pore size and sample flow rate that can improve the assay sensitivity. Secondly, we performed validation experiments to confirm the model and to characterize the effects of these two factors on the VFI's limit-of-detection with the *B. pseudomallei* assay. Lastly, a proof-of-concept experiment was conducted to detect two pathogens (*B. pseudomallei and Bacillus anthracis*) simultaneously and demonstrate the multiplexing capability of the VFI platform.

2. Materials and methods

2.1. Theoretical analysis of paper-based immuno-biosensor

Fig. 1(A) illustrates the principle of an immuno-biosensor implemented in a porous paper membrane in the vertical flow format. As the sample is pushed through the membrane vertically, the target antigens present in the sample bind to the capture antibody that have been immobilized on the membrane as a detection spot microarray. Next, as the detection antibody is flowed through, a sandwich assay is formed (as shown in Fig. 1(C)) and the labelling gold nanoparticles that are retained on the membrane generate signals for detection.

To understand the transport process, we used a simplified model to approximate the porous membrane as bundled microtubes with radius $\boldsymbol{d}$, which is equivalent to half of the pore size of the membrane materials (shown in Fig. 1(B)). The sample flows through the membrane with a flow velocity of $\vec{\boldsymbol{u}}$. The sensing length is $\boldsymbol{L_s}$, which is the detectable thickness of the membrane. The sensing area (also the imaging surface) is the top surface $\boldsymbol{A_v}$, which is a circular shape with radius $\boldsymbol{R_m}$. The internal surface of the porous structure is coated with capture antibody with a surface concentration of $\boldsymbol{\gamma}$. The diffusivity of the target is $\boldsymbol{D}$, and the antigen-antibody pair has an association constant of $\boldsymbol{k_{on}}$, and mass transfer coefficient $\boldsymbol{k_c}$.

There are two critical dimensionless numbers in the VFI system. The first one is the *Damköhler* numbers (*Da*), which characterizes the relation between adsorption rate and transport rate.

$$Da = \frac{adsorption\ \text{rate}}{transport\ \text{rate}} = \frac{k_{on}\cdot\gamma}{k_c} \tag{1}$$

The second one is the *Péclet* number (P_e), which can be used to compare convection rate and diffusion rate.

$$P_e = \frac{convection\ rate}{diffusion\ rate} = \frac{u/L_s}{D/d^2} \tag{2}$$

To capture a target antigen at low concentration, two conditions are desired:

(1) Efficient capture assay ($D_a \gg 1$), in which the rate of the antigen binding to the capture antibody is faster than the rate of antigen molecules transport to the pore wall. High flow speed increases the transport rate K_c, decreases D_a, and reduces the capture efficiency. However, this can be counterbalanced by using an assay with fast binding kinetics. In this work, we set on low concentration antigen detection with high capture antibody density, thus assuming the capture could be fast enough to ensure $D_a \gg 1$.

(2) Non-diffusion-limited assay ($P_e < 1$), which allows for all delivered antigens to diffuse to the pore wall before they are convected through the sensing area. Previous simulation showed that keeping $P_e < 1$ ensures > 90% capture efficiency [20–22]. Setting $P_e < 1$ in Eq. (2), we get the following constraint on the volumetric flow rate (Q):

$$Q < \frac{\phi D L_s A_v}{d^2} \tag{3}$$

Here ϕ is the porosity of the membrane. According to Eq. (3), decreasing the membrane pore size increases the maximum allowable

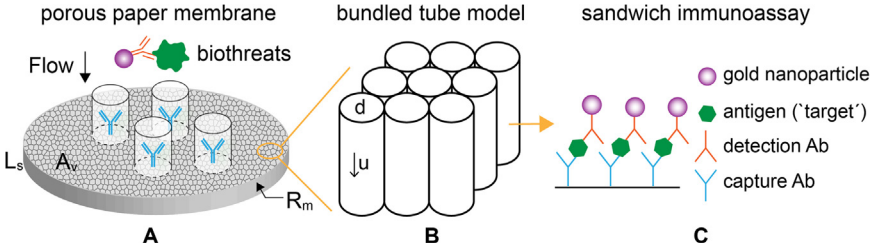

Fig. 1. (A) Principle of paper-based immunoassay in the vertical flow format. As the sample is pushed through the porous membrane, the target antigen binds to the antibody immobilized on the membrane. **(B)** Schematic of the bundled microtube model to simulate the porous membrane and the internal biomolecule transport and binding process. **(C)** Sandwich assay designed to detect the bio-threat pathogen biomarkers. The gold nanoparticles labelled with detection antibodies generate colorimetric signals as system readout.