MICRONEEDLE-BASED LATERAL FLOW IMMUNOASSAYS FOR RAPID DIAGNOSTIC TESTING

By

Xue Jiang

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ABSTRACT
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Lateral flow immunoassays (LFIAs) are one of the most commonly used tests to detect and screen for infectious diseases in the world. While these tests are simple, inexpensive and readily available, they suffer from several limitations. First, many LFIAs require a blood sample which involves trained medical personnel, poses risks of infection, and can complicate cooperation in young children and communities with blood taboos. Second, conventional LFIAs are comprised of multiple components and materials, which complicates the fabrication process and increases overall device costs. Third, LFIAs generally only provide qualitative results (i.e. positive vs. negative), which limits their diagnostic utility. To address these limitations, this research aims to investigate new approaches for developing LFIA platforms which do not require blood sampling, are simple to fabricate, and are capable of quantitative analytical measurements.

We first explored the development of a microneedle-based LFIA patch for rapid protein detection in dermal interstitial fluid. This device integrates a hollow microneedle array for minimally invasive sample extraction with a colloid gold-based LFIA. A simple gold enhancement treatment was employed to enhance the detection sensitivity of this assay, and the underlying mechanism of this enhancement mechanism was elucidated through experimental investigation. For proof of concept, this device was used to detect *Plasmodium falciparum* histidine-rich protein 2 (PfHRP2), an important biomarker for malaria infection,
in simulated interstitial fluid, which could be detected at concentrations down to 8 ng/mL. Furthermore, each test can be completed in less than 20 minutes and requires no equipment.

We also developed a unique strategy for fabricating LFIAs on a single piece of cellulose paper via laser cutting. All of the components of a conventional LFIA, such as the sample, conjugate and absorbent pads and nitrocellulose membrane, are integrated on a single-layer cellulose paper “card”. Various device parameters, such as the test strip geometry, composition of the blocking solution and amount of colloid gold-antibody conjugates, were optimized to minimize nonspecific binding of conjugates for enhanced detection sensitivity. The functionality of this assay was validated by using it to detect PfHRP2 in human plasma samples which could be detected at concentrations down to 4 ng/mL with excellent specificity.

Lastly, towards a LFIA platform capable of quantitative analytical measurements, we have integrated an electrical impedance sensor with our single layer LFIA system. This device employs interdigitated sensing electrodes and an inductive coil antenna for wireless sensing using an impedance analyzer. A unique detection scheme is employed where the electrical properties of the sensing electrodes are monitored in response to surface immobilization of gold colloid-antibody conjugates. Device testing was carried out by performing quantitative measurements of PfHRP2 in buffer samples, which could be detected at concentrations down to 8 ng/mL. In conclusion, we have presented several unique LFIA platforms that offer simplified fabrication, ease of use and improved analytical performance compared with conventional LFIAs, which can ultimately enhance the usefulness of LFIA technology for point-of-care diagnostic testing.
To my parents and my friends for their endless love and support.
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TABLE OF CONTENTS

LIST OF FIGURES ..............................................................................................................xi

1. INTRODUCTION ........................................................................................................... 1
   1.1 Point-of-Care Diagnostic Testing ........................................................................... 2
   1.2 Lateral Flow Immunoassay .................................................................................. 3
   1.3 Microneedles for POC Testing ............................................................................. 8
   1.4 Objectives ............................................................................................................. 12

2. MICRONEEDLE-BASED LATERAL FLOW IMMUNOASSAY FOR RAPID PROTEIN DETECTION ................................................................................................................. 14
   2.1 Motivation ............................................................................................................. 15
   2.2 Development of Hollow Microneedle Arrays ....................................................... 16
      2.2.1 Fabrication of Hollow Microneedle Arrays – First Generation .................. 16
      2.2.2 Fabrication of Hollow Microneedle Arrays – Second Generation .......... 17
      2.2.3 Mechanical Testing of the Microneedle Array ............................................ 18
      2.2.4 Skin Insertion Testing ................................................................................... 19
      2.2.5 Liquid Extraction Testing ............................................................................ 19
   2.3 Design of Lateral Flow Immunoassay ................................................................. 20
      2.3.1 Antibody Concentration Optimization for Gold Nanoparticles (AuNPs) Conjugation ...................................................................................................................... 20
      2.3.2 Preparation of AuNP-Conjugated IgG Antibody .................................... 20
      2.3.3 Preparation of the Conjugate Release Pad ............................................ 21
      2.3.4 Preparation of the Nitrocellulose Membrane ....................................... 21
      2.3.5 Assembly of the Lateral Flow Test Strip ............................................... 22
      2.3.6 Evaluating PfHRP2 Immunoassay Sensitivity and Specificity .......... 22
      2.3.7 Scanning Electron Microscopy of AuNP-IgG Conjugates .................. 23
      2.3.8 Absorbance Measurements ..................................................................... 23
      2.3.9 Optical Transmittance Measurements ..................................................... 23
   2.4 Skin Patch Prototype ............................................................................................ 24
      2.4.1 Assembly of the Skin Patch .................................................................... 24
      2.4.2 Proof-of-Concept Demonstration ......................................................... 24
   2.5 Results .................................................................................................................... 25
      2.5.1 Design of the Skin Patch ........................................................................... 25
      2.5.2 Skin Penetration Performance ............................................................. 29
      2.5.3 Capillary-Based Sample Collection .................................................... 31
      2.5.4 PfHRP2 Detection Sensitivity and Specificity .................................... 32
      2.5.5 Detection Sensitivity Enhancement Using Gold Enhancement Solution 34
      2.5.6 Proof-of-Concept Demonstration ......................................................... 37
   2.6 Discussion and Summary ....................................................................................... 38

   3.1 Motivation ........................................................................................................... 41
   3.2 Biochemicals and Reagents .............................................................................. 43
   3.3 Preparation of AuNPs-IgG conjugates ............................................................... 44
3.4 Materials Selection ........................................................................................................44
3.4.1 Fabrication of the LFIA Card for Substrate Selection ........................................46
3.4.2 Fusion 5 Membrane ....................................................................................................46
3.4.3 Cellulose Chromatography Paper ............................................................................51
3.5 Optimization for Design of the Single Layer LFIA Card ..........................................52
3.5.1 Second Design of the Single Layer LFIA Card .........................................................52
3.5.2 Optimization of Strip Width ......................................................................................53
3.5.3 Optimization of Blocking Solution ...........................................................................55
3.5.4 Optimization of Volume of AuNPs-conjugates .........................................................58
3.6 Evaluating PfHRP2 Immunoassay Sensitivity and Specificity ....................................59
3.7 Discussion and Summary ..............................................................................................64

4. Integration of an Electronic Sensor with our Lateral Flow Assay for Quantitative
   Protein Measurements .................................................................................................66
4.1 Motivation ......................................................................................................................67
4.2 Design of Electronic Sensing Element .........................................................................68
   4.2.1 Theory of Impedance Sensing .................................................................................68
   4.2.2 Design and Fabrication of Impedance Sensor ........................................................69
4.3 Design and Fabrication of the LFIA ...........................................................................70
   4.3.1 Preparation of AuNPs-IgG conjugates ...................................................................70
   4.3.2 Preparation of LFIA Layer ....................................................................................70
4.4 Impedance Sensor-Integrated LFIA Platform ..............................................................71
   4.4.1 Device Design and Assembly .................................................................................71
   4.4.2 Evaluating Detection Sensitivity ...........................................................................72
4.5 Results ..........................................................................................................................73
   4.5.1 Fabrication of Impedance Sensors .........................................................................73
   4.5.2 Wireless Quantitative PfHRP2 Measurements ....................................................74
4.6 Discussion and Summary ..............................................................................................76

5. Conclusions and Future Work .......................................................................................78
5.1 Summary of achievements and contributions .............................................................79
5.2 Future Work ....................................................................................................................80

BIBLIOGRAPHY .............................................................................................................82
LIST OF FIGURES

Figure 1-1 Schematic of a typical LFIA test strip. [10] ................................................................. 3

Figure 1-2 Multiplexed detection using silver nanoparticles with different sizes conjugating to monoclonal antibodies bind to specific biomarkers. [13] .......................................................... 5

Figure 1-3 a) Entire portable analytical system, b) lateral flow test strip with electrochemical sensor device and c) schematic illustration of the principle of the device. [14] ............................. 6

Figure 1-4 Two dimensional paper network format multistep assays. [15] ................................. 7

Figure 1-5 Schematic illustration of transdermal drug delivery by A) solid MNs, B) coated MNs, C) dissolving MNs, D) hollow MNs and E) swellable MNs. [18] .............................. 9

Figure 1-6 Schematic representation and actual device of three-electrode MN array for alcohol monitoring in interstitial fluids. [19] ................................................................. 10

Figure 1-7 Microprojection array for intradermal biomarker capture. [21] ............................... 11

Figure 1-8 Microneedle configurations with a) planer and b) cylinder concentric microneedle substrates. [24] ................................................................. 12

Figure 2-1 Schematic illustration of the fabrication process for generating hollow SU-8 microneedle arrays - (first generation). ................................................................. 17

Figure 2-2 Schematic Illustration of the Fabrication Process for Hollow SU-8 Microneedle Arrays-Second Approach. .................................................................................. 18

Figure 2-3 Schematic illustration of lateral flow immunoassay assembly.............................. 22

Figure 2-4 Exploded view of the patch depicting the microneedle array, components of the colloidal gold-based lateral flow immunoassay and bandage tape. ......................... 25

Figure 2-5 (A) Optical micrograph of the microneedle array at 20x magnification. Scale bar, 1 mm (B) SEM image of a single microneedle. Scale bar, 200 µm. ................................. 26

Figure 2-6 Underside (A) and topside (B) views of the assembled patch............................... 27

Figure 2-7 A) Microneedle mechanical testing result generated by MATLAB. B) Microneedle before compression test. C) Microneedle after compression test............................... 29

Figure 2-8 Skin penetration performance of the microneedle array. (A) Trypan blue staining of microneedle insertion wounds in cadaver porcine skin. Scale bar, 1 mm. (B) H&E-stained section of porcine skin penetrated by the microneedle array. The insertion wound is indicated by the blue dashed line. Scale bar, 100 µm. ................................. 30
Figure 2-9 Fluid extraction capability of the microneedle array and skin patch. (A) Sequential still frame images showing a droplet of red dye being autonomously wicked by the microneedle array. The arrows indicate the position of the liquid inside the needle. Scale bars, 500 μm. (B) Sequential still frame images showing capillary-based liquid extraction and transport using an artificial skin model. Time stamps (min:sec) are located in the upper right corners.

Figure 2-10 Assay sensitivity without gold enhancement treatment.

Figure 2-11 Sensitivity and specificity of the PfHRP2 immunoassay. (A) Test results of interstitial fluid with increasing concentrations of PfHRP2. (B) Test results of interstitial fluid with 1,024 ng/mL of PfHRP2, PfLDH or Pan-Plasmodium aldolase.

Figure 2-12 Scanning electron micrographs of nitrocellulose paper obtained by SE imaging (A) and BSE imaging (D). Scale bars, 1 μm. Scanning electron micrographs of nitrocellulose paper coated with AuNP-IgG conjugates obtained by SE imaging (B) and BSE imaging (E). Scale bars, 100 nm. Scanning electron micrographs of nitrocellulose paper coated with AuNP-IgG conjugates with gold enhancement obtained by SE imaging (C) and BSE imaging (F). Scale bars, 100 nm.

Figure 2-13 Absorbance vs. wavelength and Transmission vs. wavelength.

Figure 2-14 Proof-of-concept demonstration of the skin patch. Test results obtained from young pig cadavers dermally-injected with (A) 1,024 ng/mL of PfHRP2 or (B) PBS.

Figure 3-1 A) Fabrication process of the inkjet-printed microfluidic immunosensing strip. B) Multianalyte sensing of two immunological parameters (human and mouse IgG) and a chemical parameter (pH). [71]

Figure 3-2 First Design of Single layer LIFA card for substrate selection.

Figure 3-3 Effect of particles size with a) line width and b) line intensity on Fusion 5 membrane.[70]

Figure 3-4 Sensitivity test for detection of PfHRP2 spiked in PBS from 0 - 1,024 ng/mL by applying latex beads conjugated antibodies on control and test lines.

Figure 3-5 Sensitivity test for detection of PfHRP2 spiked in PBS from 0 - 1,024 ng/mL by applying antibodies in acidic buffer on control and test lines.

Figure 3-6 Sensitivity test for detection of PfHRP2 spiked in PBS from 0 - 1,024 ng/mL by applying treatment on control and test lines region.

Figure 3-7 Sensitivity test for detection of PfHRP2 spiked in PBS from 0 - 4,096 ng/mL for chromatography paper-based assay cards.
Figure 3-8 (A) Exploded view of the single-layer lateral flow immunochromatographic assay. The assay card is encapsulated between two pieces of self-adhesive plastic film. Images of the immunochromatographic device without (B) and with (C) the top cover. .................53

Figure 3-9 (A) Test results of samples containing 32 ng/mL of PfHRP2 in PBS using assay cards with 2 mm-, 3 mm-, 4 mm- or 5 mm-wide test strips. The top cover is removed from the card for improved visualization of the test strip. (B) Mean gray values of the background color for various-width test strips. Each bar represents the mean ± standard deviation (SD) of three measurements. (C) Signal-to-background ratios (SBRs) generated from mean gray values of the test line and background color of the test strip. Each bar represents the mean ± SD of three measurements. .................................................................55

Figure 3-10 Test results of samples containing 0 ng/mL, 32 ng/mL and 1,024 ng/mL of PfHRP2 in PBS using assay cards treated with blocking solution containing (A) StabilCo® immunoassay stabilizer, (B) 5% (w/v) milk powder, (C) 1% (w/v) casein or (D) 2% (w/v) BSA. The top cover is removed from the card for improved visualization of the test strip. (E) Mean gray values of the background color of test strips treated with different blocking solutions. Each bar represents the mean ± SD of three measurements. (F) SBRs generated from mean gray values of the test line and background color of the test strips. Each bar represents the mean ± SD of three measurements...............................................................57

Figure 3-11 Test results of samples containing 16 ng/mL (A) or 1,024 ng/mL (B) of PfHRP2 in PBS using assay cards with 3 µL, 5 µL, 8 µL or 10 µL of AuNP-IgG conjugate solution. SBRs obtained from mean gray values of the test line and background color of the test strips at 16 ng/mL (C) and 1,024 ng/mL (D) of PfHRP2. Each bar represents the mean ± SD of three measurements...............................................................58

Figure 3-12 Test results of samples containing PfHRP2 from 0 to 1,024 ng/mL in PBS. Mean gray values of the test line are obtained from images in panel A. Each data point represents the mean ± SD of three measurements...............................................................60

Figure 3-13 Test results of samples containing PfHRP2 from 0 to 1,024 ng/mL in human plasma. Mean gray values of the test line are obtained from images in panel A. Each data point represents the mean ± SD of three measurements...............................................................61

Figure 3-14 (A) Test results of samples containing PfHRP2, PfLDH or pan-aldolase in PBS, and non-spiked PBS. (B) Mean gray values of the test line obtained from images in panel A. Each bar represents the mean ± SD of three measurements...............................................................63

Figure 3-15 (A) Test results of samples containing 1,024ng/mL PfHRP2, PfLDH or pan-aldolase in human plasma, and non-spiked human plasma. (B) Mean gray values of the test line obtained from images in panel A. Each bar represents the mean ± SD of three measurements...............................................................64

Figure 4-1 Schematic illustration of the impedance sensing region A) without target antigen and AuNP-labeled antibodies, and B) with antigen and AuNP-labeled antibodies. ...............68
Figure 4-2 (A) Design of a single impedance sensor, scale bar, 5mm. (B) Mask for fabricating comb fingers. and (C) mask for fabricating the coil antenna and connection between fingers.

Figure 4-3 Exploded view of the integrated LFIA platform depicting the impedance sensor, the colloidal gold-based lateral flow immunoassay and self-adhesive tape.

Figure 4-4 Experimental setup for measuring the cutout testing region on impedance sensor with wireless measurement set up.

Figure 4-5 (A) Thermal evaporated gold impedance sensors. (B) First step mask with its holder and (C) second step mask for thermal evaporation process.

Figure 4-6 (A) Impedance vs. frequency and (B) phase change vs. frequency curves for PBS samples containing varying concentrations of PfHRP2.

Figure 4-7 (A) Impedance vs. frequency and (B) phase change vs. frequency curves after signal processing for samples spiked with varying concentrations of PfHRP2.

Figure 5-1 Exploded view of the integrated skin patch depicting the microneedle array, the colloidal gold-based lateral flow immunoassay and bandage tape.
1. INTRODUCTION
1.1 Point-of-Care Diagnostic Testing

Infectious diseases are one of the leading causes of morbidity and mortality in the developing world [1]. In 2017, there were ~3 million deaths due to tuberculous, HIV/AIDS and malaria, most of which occurred in low- and middle-income countries [2]–[4]. Most infectious diseases in the developing world are treatable, however, one of the main roadblocks to disease control and elimination is the lack of simple, rapid and low cost tools for diagnostic testing at early stages of infection [5]. Traditional diagnostic technologies requires highly regulated laboratories and quality-assessed environment, which cannot address the needs of people with poorly resourced healthcare facilities [6]. Not only for the detection of the infectious diseases, but also for patients with other diseases that requires continuously therapeutics monitoring or early disease detection, the cost of visiting the health professionals continuously will become the financial burden of their families.

In late 1970s, the development of microfluidics showed huge potential in integrating microfluidic technology with point-of-care (POC) devices to process and analyze samples on a single device [7]. Researchers have developed numerous POC devices in recent decades. Common applications for POC devices include the detection of proteins, metabolites and other small molecules, nucleic acids, human cells, microbes/pathogens, and drugs [8]. Paper-based diagnostic devices play a remarkable in the development of POC devices due to its advantages of low-cost, disposability and simplicity. Various types of paper membrane available in the market also contribute to the development of paper-based diagnostic devices for POC application. Lateral flow immunoassays, discussed in the next section, play an important role in the development of paper-based POC devices.
1.2 Lateral Flow Immunoassay

While laboratory-based diagnostic tests, such as microscopy and molecular assays (polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA)) exist for most infectious diseases, they are laborious, time-consuming, expensive, and rely on specialized equipment, making them ineffective for routine use in resource-limited settings. Rapid diagnostic tests (RDTs), such as lateral flow immunochromatographic assays, are simple, inexpensive and readily available, making them promising tools for disease detection and screening in developing countries.

Lateral flow immunoassays (LFIA) are an extension of latex agglutination tests that were developed by Singer and Plotz in 1956 [9]. LFIA tests are commonly used in POC medical diagnostic devices [10]. A standard layout of a LFIA device is as shown in Fig. 1-1.

![Figure 1-1 Schematic of a typical LFIA test strip. [10]](image)

The samples pad is used to modulate the chemicals or analytes to reduce the variability. Samples are expected to be evenly released to the conjugate pad with the adjusted properties. The sample pad is also used to adjust pH values which influences the ionic condition, collect the unexpected coarse materials such as separating red blood cells
and plasma, and reduce the flow rates to maximize the reaction time when mixing with the materials on the conjugate release pad. The sample pad is normally made from glass fiber, 100% cotton linter or other filtration membranes [11]. The absorbent pad is commonly be made from the same material as the sample pad, and its main function is to keep the forward movement of the liquid and to absorb all the waste solution in the system to prevent any inaccurate results caused by liquid backflow.

The conjugate release pad is used to store the microspheres/microparticles/fluorescence-labeled antibodies. Labeled antibodies are dried on the conjugate pad, solubilized when the sample passing through, and released to the reaction membrane. Blocking treatment is necessary before the application of the labeled antibodies to reduce the non-specific binding between the antibodies and pad surface. The most commonly used treatment solutions are the mixture of borate buffer (pH 7.0-7.5) supplemented with 1% (w/v) to 10% (w/v) sucrose or trehalose mixed with 1% (w/v) or 2% (w/v) bovine serum albumin (BSA) buffer. Other treatment solutions such as 1% (w/v) casein in PBS, 5% (w/v) milk, whole sera, gelatin and some polymer-based solutions can also be used to replace the use of BSA for various applications. Additional nonionic surfactants, such as Tween-20 or Triton X-100 solutions, can be applied to further reduce the non-specific binding. The sucrose or trehalose solution can help to coat the antibodies to protect the protein structure during the drying process. The conjugate pad is normally made in glass fiber or polyester.

Reaction membranes are used to facilitate as a capturing mechanism with the immobilized antibodies on test and control lines. The immobilized antibodies can capture the analytes that are specific to them and the labels on them can show the color/florescence
indicating the presence of the analytes. Additional blocking treatment may be necessary to prevent false positive results due to non-specific binding of antibody conjugates. However, excess blocking may also cause loss of detection sensitivity. Reaction membranes are commonly made from nitrocellulose, nylon or polyvinylidene fluoride. The wide selection of membrane materials with different flow rate and pore size provides researchers with plenty of choices for different applications [12].

The backing material of the LFIA system prevents the vertical flow in the device and also provide rigidity to easily handle the strips. The backing material is normally self-adhesive, so all the components can be easily assembled together. However, to reduce the contact issue between different components, additional laminated layers are applied for commercialized LFIA devices, which can also prevent the surface damage or human contacts of the chemicals in the strips [12].

Figure 1-2 Multiplexed detection using silver nanoparticles with different sizes conjugating to monoclonal antibodies bind to specific biomarkers. [13]
While most LFIs only detect a single analyte, they can also be made to detect multiplex targets on a single strip. Dr. Lee Gehrke’s group at Massachusetts Institute of Technology developed an LFIA for multiplexed detection of dengue virus NS1 protein, Yellow Fever Virus NS1 protein, and Ebola virus, Zaire strain glycoprotein GP. This device consists of varying sized silver nanoparticles showing different colors conjugated to monoclonal antibodies that bind to specific biomarkers. As shown in Fig. 1-2, they used this device to successfully detect three different biomarkers at concentrations down to 150 ng/mL in a single test strip [13].

Figure 1-3 a) Entire portable analytical system, b) lateral flow test strip with electrochemical sensor device and c) schematic illustration of the principle of the device. [14]
Although most LFIA tests only indicate the existence or absence of target analytes, quantitative results can be achieved by either analyzing the intensity of the test line(s) or incorporating an electrical sensor. Dr. Yuehe Lin’s group at the Pacific Northwest National Laboratory has developed an integrated system of LFIA test strip with electrochemical sensor for quantification of phosphorylated cholinesterase (biomarker of exposure to organophosphorus (OP) agents), as shown in Fig. 1-3. The baseline for the calculation of acetylcholinesterase (AChE) inhibition in this system exploits the reactivation of the phosphorylated AChE enzyme to enable the measurement of the total amount of AChE. Quantitative measurement of the phosphorylated adduct can be calculated by the differences between the active AChE with total AChE. This system successfully shows the linear response between the AChE enzyme activity and enzyme concentration from 0.05 to 10 nM with a lower detection limit of 0.02 nM [14].

Figure 1-4 Two dimensional paper network format multistep assays. [15]
Due to the simplicity of the LFIA tests, they are often limited by a single chemical delivery step. To achieve multistep chemical processing, Dr. Paul Yager’s group at the University of Washington has demonstrated a two-dimensional system based on the conventional LFIA test to detect the PfHRP2 protein biomarkers as shown in Fig. 1-4. Their two-dimensional paper network sequential delivery of multiple reagents with a signal amplification reagent resulted in highly sensitive detection of PfHRP2 down to 5 ng/mL.

1.3 Microneedles for POC Testing

The development of microneedle (MN) arrays started from 1970s [16], and MN arrays were used for transdermal transport of macromolecules or possibly microparticles in 2004 [17]. Due to its minimally invasive nature, MN arrays can painlessly pierce the epidermis layer and create pathways for drug to be diffused under skin by dermal microcirculation. Various types of MN arrays were developed in recent years including solid MNs, coated MNs, dissolving MNs, hollow MNs and swellable MNs [18]. A schematic illustration of five different MN types used in transdermal drug delivery is shown in Fig. 1-5.
Figure 1-5 Schematic illustration of transdermal drug delivery by A) solid MNs, B) coated MNs, C) dissolving MNs, D) hollow MNs and E) swellable MNs. [18]

Solid MNs penetrate the skin first and then apply drug through the opening on the epidermis. Coated MNs will leave the coated layer of drug in the skin when inserting into skin. Dissolving MNs dissolve entire MNs inside the skin and only the substrate is removed. Hollow MNs deliver the drug through the lumens of the microneedles. Swellable MNs absorb the interstitial fluid (ISF) in the epidermis and swell to release the drug inside those MNs. The common material for making these swellable MNs is hydrogel. MN arrays are commonly made from silicon, metals, polymers, ceramics, silica glass or carbohydrates. Polymers are mainly used to produce dissolving/biodegradable and swellable MN arrays and new studies have using polymers for fabrication of solid, hollow and coated MNs. Recently, polymers drawing increasing attention due to their excellent biocompatibility, biodegradability, low toxicity and low cost.
Figure 1-6 Schematic representation and actual device of three-electrode MN array for alcohol monitoring in interstitial fluids. [19]

In addition to drug delivery, MN arrays are being increasingly used for POC diagnostic applications. Dr. Joseph Wang’s group at the University of California, San Diego demonstrated an alcohol monitoring system using MN sensor arrays (Fig. 1-6). The hollow MN array was fabricated by scalable micro-injection molding techniques, and three-electrode system integrating by platinum and silver wires inserted into the lumens of the MNs. The matrix of alcohol oxidase and chitosan with a Nafion outer layer was immobilized on the platinum working electrode inside the lumen of one of the MN, which reacts with the ethanol in the interstitial fluid. [19] Using a similar approach, they have recently demonstrated another MN biosensor for transdermal detection of nerve agents with a modified carbon-paste array electrode transducer. [20]
Figure 1-7 Microprojection array for intradermal biomarker capture. [21]

Dr. Mark A. Kendall’s group at the University of Queensland in Australia has developed a surface modified solid microprojection/microneedle arrays for intradermal biomarker capture. The MN array was fabricated from silicon by using deep reactive ion etching (DRIE), and was subsequently coated with gold for enhanced protein immobilization performance. With a layer of polyethylene glycol (PEG) and covalently bonded capture protein, the MN array could successfully measure the anti-FluVax® IgG by using the extracted ISF from the skin. [21] Using a similar approach, they have also developed a MN skin patch for the detection of PfHRP2 [22] and dengue virus NS1 protein [23].

The majority of the MN array systems for diagnostic testing employ solid MNs for *in vivo* electrochemical measurements. However, the fabrication of electrochemical sensors on MNs is challenging. An alternative approach is to use hollow microneedle arrays to extract blood or dermal interstitial fluids for subsequent detection. Dr. Phillip R. Miller and his group have discovered that dermal ISF can be extracted using a single hollow
microneedle to minimize dermal compaction at insertion sites [24]. Using this device, about 20 μL – 70 μL ISF can be collected by a single hollow microneedle. The discovery of this configuration provides more possibility for using hollow MN arrays for blood-free POC diagnostic devices.

Figure 1-8 Microneedle configurations with a) planer and b) cylinder concentric microneedle substrates. [24]

1.4 Objectives

Although LFIA technology remains one of the most commonly used tests for diagnostic testing in the world, the need for blood-sampling, lack of quantitative measurements and complicated fabrication process hinders its utility for many diagnostic applications. Therefore, the aim of this research is to investigate the development of new lateral flow immunoassays for diagnostic testing, which is low cost, simple to fabricate, does not require blood sampling, and offers quantitative protein measurements. To achieve this goal, we have pursued the following objectives:

Objective 1: Develop a microneedle-based lateral flow immunoassay for rapid protein detection;

Objective 2: Develop a single layer lateral flow immunochromatographic assay;
Objective 3: Integrate an electronic sensor with our single-layer lateral flow immunoassay for quantitative protein measurements.

In this dissertation, we present the development of several unique lateral flow immunoassay technologies. The first is a microneedle-based LFIA skin patch for blood-free protein detection (Objective 1) presented in Chapter 2. Next, to simplify the fabrication process of conventional LFIA s, we demonstrate a novel design and fabrication process to create a single layer LFIA card (Objective 2) presented in Chapter 3. Lastly, we present our efforts to develop a LFIA capable of quantitative measurements (Objective 3) presented in Chapter 4. In Chapter 5, we conclude by highlighting our achievements and discussing the future direction of this research. The LFIA systems presented in this dissertation represents a culmination of the experiences and knowledge that I have obtained throughout my entire graduate study. It also provides a new direction for transforming traditional RDTs requiring blood sampling to ones that are painless, blood-free, simpler to fabricate and can provide enhanced diagnostic information.
2. MICRONEEDLE-BASED LATERAL FLOW IMMUNOASSAY FOR RAPID PROTEIN DETECTION
2.1 Motivation

RDTs are the most commonly used tests for detecting malaria infection in malaria-endemic regions. However, RDTs and laboratory-based methods rely on blood sampling, which requires trained medical personnel and poses risks of infection or accidental disease transmission [25]. Furthermore, the invasive nature of blood sampling can complicate cooperation, especially in young children, asymptomatic individuals who are disinclined to be tested, and communities with blood taboos [26]. Efforts toward a blood-free diagnostic test for malaria have resulted in a urine dipstick for the detection of PfHRP2 [27], [28]; however, its moderate sensitivity limits its usefulness for routine diagnostic testing. While PfHRP2 has also been detected in the saliva of malaria patients using ELISA [26], [29], its concentration is ~20× lower than in blood, which is well below the detection limit of malaria RDTs.

In the past few decades, much attention has been directed at the use of microneedles for minimally invasive transdermal drug and vaccine delivery [30]. Compared to hypodermic needles, microneedles avoid the nerves and vascular structures located in the deeper layers of the dermis thereby significantly minimizing their associated pain and risks of infection [31]. Recently, the utility of microneedles for minimally invasive transdermal biosensing has also been reported [32]. Solid microneedles have been used for transdermal extraction of glucose and protein biomarkers, including Dengue virus nonstructural protein-1 and PfHRP2, in mice [21]–[23], [33]. While these platforms are capable of selective extraction of analyte, they require manual processing (e.g. desorption, pipetting, centrifugation) to recover, concentrate and analyze biomarkers. Microneedle-based electrochemical sensors for measuring metabolites, such as glucose and lactate, in dermal
interstitial fluid have been demonstrated [34]–[37]; however, these platforms rely on electronic components (e.g. detectors, power source), which increases their size, complexity and costs. Despite the recent progress made in microneedle-based biosensors, their current reliance on laborious sample processing or electronics makes them poorly suited for use in resource-limited settings.

In this Chapter, we report a microneedle-based biosensing platform for rapid, colorimetric detection of protein biomarkers. This novel device integrates a hollow microneedle array with a colloid gold-based lateral flow immunoassay on a disposable skin patch. *Pf*HRP2 was used as target analyte in this project to test the performance of the integrated system.

### 2.2 Development of Hollow Microneedle Arrays

#### 2.2.1 Fabrication of Hollow Microneedle Arrays – First Generation

Microneedle arrays were fabricated from polymerized SU-8 on a polyethylene terephthalate (PET) film, as shown in Fig. 2-1. Briefly, 100-µm layers of polydimethylsiloxane (PDMS) (Dow Corning) was deposited and cured at 80°C for 1.5 hours on both sides of PET film (McMaster-Carr). The sandwiched structure was attached on a glass wafer. 50-µm holes were generated in the PDMS-PET-PDMS assembly using a CO₂ laser cutter (Universal Laser System), followed by 100 nm gold deposition. 1,000 µm of SU-8 2025 (MicroChem) was coated the wafer and soft baked at 95 °C for 12 hours, followed by 6000 mJ backside UV exposure (365nm) with a 900 µm spacing between wafer and custom photomask designed using AutoCAD (Autodesk). The polymerized SU-8 substrate was baked at 55°C for 1.5 hours, placed in a 1-methoxy-2-propanol acetate solution (MicroChem) for 3 hours with gentle agitation, followed by ultrasonication for 20
minutes. Microneedle arrays were rinsed in isopropanol and deionized water, dried under a stream of purified N₂ and left at room temperature overnight to fully dry. Arrays were exposed to a UV/Ozone treatment (Novascan Technologies) for 20 minutes to make the surfaces hydrophilic.

![Figure 2-1 Schematic illustration of the fabrication process for generating hollow SU-8 microneedle arrays - (first generation).](image)

**2.2.2 Fabrication of Hollow Microneedle Arrays – Second Generation**

A schematic of the fabrication process for the second-generation hollow microneedle arrays is shown in Fig. 2-2. A 100 μm-thick layer of PDMS was spin-coated onto a 100 μm-thick PET film and soft baked at 80°C for 1.5 hours. Two separate PDMS-PET assemblies were prepared. On one of the PDMS-PET assemblies, 50-μm holes configured in a 4 × 4 array were generated using a CO₂ laser cutter. The other assembly was attached to the PET-side of the laser-cut assembly forming a 4-layer PDMS-PET structure. 100 nm of gold was thermally evaporated on the topside of the 4-
layer assembly, followed by the subsequent removal of the top PDMS layer. 1,000 µm of SU-8 2025 was spin-coated on the 3-layer assembly, baked at 95 °C for 12 hours, followed by 6000 mJ backside UV exposure (365 nm) through customized photomask.

A 900 µm gap distance between the PET-PDMS-PET assembly and photomask resulted in generation of tapered microneedles. The polymerized SU-8 substrate was baked and developed as described in Section 2.2.1. Microneedle arrays were rinsed, dried and exposed to a UV/Ozone treatment prior to use.

2.2.3 Mechanical Testing of the Microneedle Array

The mechanical behavior of the SU-8 microneedles was tested using a Dynamic Mechanical Analysis machine (TA Instrument). For each measurement, a 4 × 4 microneedle array was affixed to the bottom plate using double-sided tape and compressed by the upper plate from 0 to 17 N at a rate of 0.5 N/min. Force-displacement curves were
generated using four different arrays and plotted as the mean data ± standard deviation (SD). The exported data was normalized and plotted by MATLAB (MathWorks).

2.2.4 Skin Insertion Testing

Cadaver porcine skin was obtained from the MSU Meat Lab, rinsed in deionized water, shaved and trimmed of fat using a scalpel (Cancer Diagnostics, Inc.). A 5 cm × 5 cm section was cut and secured to a wax block using pin-needles. Microneedle arrays coated in a 0.4% Trypan Blue solution (Sigma Aldrich) were pressed into the skin using a thumb, and removed after 5 minutes. Histological analysis was performed by soaking the skin sample and paraffin wax block in a 10% formalin solution (Cancer Diagnostics, Inc.) for 48 hours, cutting 500 μm sections, followed by fixing in paraffin, drying, cutting into 2-micron slices and staining with hematoxylin and eosin (H&E) (Sigma-Aldrich, MO). The morphology of the penetration holes was imaged and captured using a Nikon Eclipse LV100ND microscope and Nikon Digital Sight DS-Fi2 camera.

2.2.5 Liquid Extraction Testing

Liquid extraction testing of UV/Ozone-treated microneedles was carried out by lowering the microneedle array into a droplet of red dye solution. The microneedle array was affixed to the manually controlled stages, and the stage was slowly lowered until the microneedle tips contacted the droplet. Video recordings of fluid extraction were captured using a Nikon SMZ745T stereoscopic microscope Nikon Digital Sight DS-Fi2 camera. We assessed the capacity of the skin patch to autonomously collect interstitial fluid using an artificial skin model. Briefly, 2% (w/v) agar gel (Sigma Aldrich) solution was boiled, poured into a 100-mm petri dish and cured at 4°C overnight. Colored solution was dispensed on top of the agar gel, and covered by Parafilm (Bemis Company, Inc.), which
was carefully stretched over the petri dish to ensure the removal of air bubbles. To initiate the test, the patch was placed on the agar-Parafilm assembly and gently pressed using the blunt end of a tweezer. Video recordings were captured using an iPhone XS. Post processing (i.e. frame extraction) of video recordings were performed using Windows Live Movie Maker software (Microsoft).

2.3 Design of Lateral Flow Immunoassay

2.3.1 Antibody Concentration Optimization for Gold Nanoparticles (AuNPs)

Conjugation

Ten 200 μL of 30 nm OD 1 colloid gold solutions (Sigma Aldrich) were prepared in separate tubes. Solutions of 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6 and 1.8 μg mouse anti-
\textit{Pf}HRP2 IgG (ICL, Inc.) were sequentially applied to each tube, vortexed for 30 seconds, gently agitated for 30 minutes and incubate at room temperature for 20 minutes. Solutions of 20 μL of 10% (w/v) Sodium Chloride (NaCl) were added to each tube and incubated at room temperature for another 20 minutes. If the AuNPs are not sufficiently conjugated with antibodies, adding NaCl solution will cause the aggregation of AuNPs that leads to color changes of the solution. During the first sets of experiment, color stays the same for the tube with 1.6 μg of antibodies added. Next, by following the same procedure, solutions of 1.42, 1.44, 1.46, 1.48, 1.50, 1.52, 1.54, 1.56 and 1.58 μg mouse anti-
\textit{Pf}HRP2 IgG were sequentially added to nine 200 μL of 30 nm OD 1 colloid gold solutions. It turned out that adding 1.52 μg antibodies are the optimized amount.

2.3.2 Preparation of AuNP-Conjugated IgG Antibody

1 mL of 30 nm colloid gold solution was prepared at a concentration of $1.8 \times 10^{11}$ particles/mL. A solution of 0.82 μL mouse anti-
\textit{Pf}HRP2 IgG at a concentration 9.28
mg/mL was added to the AuNP solution, vortexed for 30 seconds, gently agitated for 25 minutes and incubated at room temperature for 20 minutes. The mixture was transferred to a new tube containing 7.5 mg of BSA powder, sonicated for 15 seconds, gently agitated for 25 minutes and incubated at room temperature for 20 minutes. The mixture was centrifuged at 5,000 g for 20 minutes, the supernatant was removed, and the precipitate was resuspended in 200 μL of StabilBlock® Immunoassay Stabilizer (Surmodics, Inc.) with 0.25% (w/v) Tween-20. The IgG-AuNP solution was stored at 4°C overnight prior to use.

2.3.3 Preparation of the Conjugate Release Pad

Untreated glass fiber strips (EMD Millipore) were soaked in a solution containing 10% (w/v) sucrose (Sigma Aldrich), 2% (w/v) bovine serum albumin (BSA) (Sigma Aldrich) and 0.25% (w/v) Tween-20 (Sigma Aldrich) mixture for 1 hour and dried at 37°C for 2 hours. Strip were cut into 5 mm × 3 mm pads using a guillotine cutter (BioDot), treated with 5 μL of AuNP-conjugated IgG solution, and dried at 37 °C for 2 hours.

2.3.4 Preparation of the Nitrocellulose Membrane

A 25 mm-wide FF120HP PLUS nitrocellulose membrane (GE Healthcare) was cut into 300-mm long strips using a guillotine cutter. A solution of 200 μg/mL rabbit anti-mouse IgG H&L (Abcam) and 800 μg/mL of mouse anti-PfHRP2 IgM (ICL, Inc.) were dispensed on the nitrocellulose membrane strips as control and test lines, respectively, using an automated liquid dispensing platform (BioDot). The nitrocellulose membrane card was incubated at 37 °C for 2 hours and cut into 3 mm-wide strips.
2.3.5 Assembly of the Lateral Flow Test Strip

Figure 2-3 Schematic illustration of lateral flow immunoassay assembly.

A schematic of the lateral flow test strip is shown in Fig. 2-3. A 10 mm × 10 mm cellulose absorbent pad (EMD Millipore) was attached to one side of the card with a ~3 mm overlap with the nitrocellulose membrane. The conjugate release pad and an 8 mm × 8 mm glass fiber sample pad (EMD Millipore) were sequentially attached to the other side of the card with ~3 mm overlaps with the nitrocellulose membrane and conjugate pad, respectively. The absorbent and conjugate pads were cut from stock sheets using a CO₂ laser cutter. Sample, absorbent and conjugate pads were affixed to the nitrocellulose membrane using medical grade double-sided adhesive tape (3M). An 8 mm × 8 mm opening was generated in the adhesive tap under the sample pad using a CO₂ laser cutter to provide access for the microneedle array.

2.3.6 Evaluating PfHRP2 Immunoassay Sensitivity and Specificity

Recombinant PfHRP2 antigen (CTK Biotech) was serially diluted in dermal interstitial fluid simulant, which was prepared based on prior recipes [38], [39] with minor modifications. Briefly, a solution of 2 mg/L human serum albumin (Abcam) was dissolved in 100 mL of Tyrode's solution (Sigma-Aldrich). For each measurement, 15 μL of sample was dispensed on the sample pad, followed by 30 μL of PBS, which served as a flushing agent. After 5 minutes, 5 μL of GoldEnhance™ Blots solution (Nanoprobes, Inc.) was
applied in the middle of the test and control lines. For assay specificity testing, measurements were performed using dermal interstitial fluid simulant spiked with 1,024 ng/mL of PfHRP2, Pan-Plasmodium aldolase (CTK Biotech) or Plasmodium falciparum lactate dehydrogenase (PfLDH) (CTK Biotech). Images of the test results were obtained after 15 minutes using a CanoScan 9000F scanner (Canon).

2.3.7 Scanning Electron Microscopy of AuNP-IgG Conjugates

Scanning electron micrographs of the test line of lateral flow immunoassays were obtained using a JEOL 7500F scanning electron microscope to characterize the morphology of AuNP-IgG conjugates with and without gold enhancement treatment. Lateral flow test strips were tested using 1,024 ng/mL of PfHRP2, treated with 5 µL of GoldEnhance™ Blots solution, rinsed in deionized water, dried in a dehumidifier chamber overnight and coated with 5 nm of osmium prior to imaging.

2.3.8 Absorbance Measurements

Absorbance spectra of AuNP-IgG conjugate solution with and without GoldEnhance™ Blots solution were obtained using a NanoDrop UV-Vis spectrophotometer (Thermo Scientific, MA). AuNP-IgG conjugate solution was prepared as described in Section 2.3.3 above. Gold enhancement treatment was carried out by centrifuging 500 µL of AuNP-IgG conjugate solution at 5,000 g for 10 minutes, removing 250 µL of supernatant, and resuspending the precipitate in 250 µL of GoldEnhance™ Blots solution.

2.3.9 Optical Transmittance Measurements

Transmittance spectra of nitrocellulose paper coated with AuNP-IgG conjugates treated with and without gold enhancement solution were obtained using a Perkin Elmer
Lambda-900 UV-Vis-NIR spectrometer with an integrating sphere. Transmittance spectra of unmodified nitrocellulose paper were also obtained and used as a blank control. Nitrocellulose paper was cut into 20 mm × 20 mm pieces using a CO$_2$ laser cutter and immersed in AuNP-IgG conjugate solution with or without GoldEnhance™ Blots solution for 5 seconds and dried in a dehumidifier chamber for 1 hour prior to measurements.

2.4 Skin Patch Prototype

2.4.1 Assembly of the Skin Patch

The skin patch was assembled by first attaching the microneedle array to an 8 mm × 8 mm piece of Supor®-5000 hydrophilic membrane (Life Sciences) which was subsequently attached to the backside of the sample pad of the lateral flow test strip. The microneedle array-lateral flow test strip assembly was affixed to a BAND-AID©-sized piece of Nexcare™ tape (3M) containing cutouts for the buffer port, result window, and test (“T”) and control (“C”) line, which were generated using a CO$_2$ laser cutter. A piece of transparent Nexcare™ tape, containing a cutout for the microneedle array, was affixed to the underside of the device to fully enclose the lateral flow test strip and secure it within the patch. The self-adhesive backing of the tape allows the patch to remain in place on the skin during testing.

2.4.2 Proof-of-Concept Demonstration

One-week old pig cadavers were obtained from a Michigan State University swine farm. A 30 mm × 30 mm section of the cadaver was rinsed with deionized water and shaved using a razor. Interstitial fluid simulant spiked with 1,024 ng/mL of PfHRP2 or PBS was dermally injected using a syringe pump (BD Scientific). The patch was applied the skin and pressed gently to initiate interstitial fluid extraction. After 17 minutes, 30 μL of PBS
was dispensed into the buffer port. Photographs of the test results were obtained using an iPhone XS.

2.5 Results

2.5.1 Design of the Skin Patch

The device consists of a hollow polymeric microneedle array integrated with a colloidal gold-based lateral flow immunoassay encased within a self-adhesive patch (Fig. 2-4). The microneedle array is comprised of hollow microneedles made from polymerized SU-8 photoresist on a flexible PET substrate.

Figure 2-4 Exploded view of the patch depicting the microneedle array, components of the colloidal gold-based lateral flow immunoassay and bandage tape.

Polymerized SU-8 exhibits excellent biocompatibility with no cytotoxicity effects and minimal interaction in tissue [40], making it well suited for \textit{in vivo} skin insertion. Microneedles were designed to safely penetrate human skin with minimal pain and extract dermal interstitial fluid for subsequent protein detection. Dermal interstitial fluid is located in the dermis, which is situated directly beneath the epidermis. The preferred application
site for the patch is the upper anterior forearm due to its ease of access and lack of body hair [41]. The thickness of the skin in the forearm ranges from 800 – 1,000 µm [42]. Studies to evaluate the effect of microneedle design on pain in humans [31] showed that microneedles shorter than 700 µm resulted in significantly less pain compared with a 26-gage hypodermic needle. Furthermore, sharper microneedles (tip angles ≤ 20°) generated the least amount pain compared with microneedles with tip angles > 20°, and there was no significant difference in pain for 700 µm-long needles having widths of 160, 245 or 465 µm.

Figure 2-5 (A) Optical micrograph of the microneedle array at 20× magnification. Scale bar, 1 mm (B) SEM image of a single microneedle. Scale bar, 200 µm.

Based on these results, we designed our microneedles to be 700 µm in length with a base diameter of 400 µm and hole diameter of 80 µm. The hole is offset 80 µm from the central axis of the needle resulting in a sharp lancet point geometry with a tip angle of ~14° (Fig. 2-5A). This microneedle design enables dermal interstitial fluid to be extracted from the anterior forearm, while minimizing its pain. Microneedles are configured in a 4 × 4, two-dimensional array to increase the rate of sample extraction, with a needle-to-needle
spacing of 2 mm (Fig. 2-5B). The overall size of the array is 7 mm × 7 mm, resulting in a miniature device footprint.

Figure 2-6 Underside (A) and topside (B) views of the assembled patch.

The microneedle array is attached to the lateral flow test strip, which is based on a conventional lateral flow immunochromatographic assay consisting of a cellulose sample pad, glass fiber conjugate release pad, cellulose absorbent and nitrocellulose membrane on a polyvinyl chloride backing card. The conjugate release pad contains anti-P/HRP2 IgG antibodies labeled with gold nanoparticles (AuNPs), and the nitrocellulose membrane contains immobilized anti-P/HRP2 IgM antibodies and anti-mouse IgG H&L antibodies representing the test line and control line, respectively. Upon applying the patch to the skin, the microneedles penetrate the skin and extract dermal interstitial fluid via surface tension. The sample region is designed to allow the extracted interstitial fluid to be autonomously
transported from the microneedles to the backside of the sample pad via a hydrophilic membrane and subsequent passage through the lateral flow test strip. After a ~15-minute sample collection period, a drop of saline solution (PBS) is dispensed in the buffer port. If \( PfHRP2 \) is present in the sample, it will bind to AuNP-IgG antibodies and migrate towards the test line where they will be captured to generate a red line (denoting a positive result). Uncaptured AuNP-IgG antibodies will subsequently bind to the control line to generate a second red line, verifying the test result. If the sample does not contain \( PfHRP2 \), the AuNP-IgG antibodies will only bind to the control line and generate a single red line (denoting a negative result).

The microneedle array-lateral flow test strip assembly is sandwiched between two layers of medical grade Nexcare™ tape (top layer is opaque and the bottom layer is transparent), as shown in Fig. 2-6A. The underside of the patch contains a cutout for the microneedle array, and allows the entire assembly to be fully encased within the patch, eliminating potential hazards associated with reagent leakage. The topside of the patch contains cutouts for the buffer port, result window, and test (“T”) and control (“C”) line indicators (Fig. 2-6B). The self-adhesive backing of the patch allows it to remain in place on the skin during testing.
2.5.2 Skin Penetration Performance

![Figure 2-7](image)

Figure 2-7 A) Microneedle mechanical testing result generated by MATLAB. B) Microneedle before compression test. C) Microneedle after compression test.

We first characterized the mechanical strength of the microneedle array to determine its capacity for skin penetration. Force-displacement curves of the microneedle arrays subjected to mechanical compression are shown in Fig. 2-7A. Based on this data, our microneedle array exhibits a yield strength of 17 ± 1 N, which is ~13-fold larger than the force required to penetrate human skin (0.08 N/needle) [43]. These results indicate that our microneedle array has a safety factor of 12.5, ensuring that the microneedles will not break during skin penetration. Scanning electron microscopy (SEM) images of individual microneedles obtained before (Fig. 2-7B) and after (Fig. 2-7C) compression testing show that the microneedles undergo plastic deformation at higher forces (> ~16 N), but do not break, eliminating potential risks associated with needle failure.
Next, we assessed the skin penetration capability of our microneedle array using porcine skin, which is anatomically and biochemically similar to human skin [44]. As shown in Fig. 2-8A, a confined insertion wound is generated by each microneedle with no impact to the surrounding skin. Histological analysis was also performed to evaluate the effect of microneedle penetration in skin tissue. As shown in the H&E stained section of porcine skin (Fig. 2-8B), each insertion site is characterized by a conical penetration cavity which pierces through the epidermis. The depth of the cavity is ~450 μm, which permits extraction of dermal interstitial fluid, while avoiding the nerves and vascular structures located in the deeper layers of the dermis. These results validate the effectiveness of our microneedle array in safely penetrating skin for interstitial fluid sampling and suggest that it will cause no bleeding and minimal pain in humans.
2.5.3 Capillary-Based Sample Collection

While polymerized SU-8 offers excellent biocompatibility and exceptional mechanical properties advantageous for skin penetration, its mild hydrophobicity (water contact angle ~80°C) [45] hinders its capacity for capillary-driven liquid transport. Therefore, the surfaces of the microneedle array were made hydrophilic through a UV/Ozone surface treatment to facilitate sample collection and transport. UV/Ozone exposure for 20 minutes was shown to dramatically improve the surface wettability of SU-8 (water contact angle ~22°) [46]. After fabrication of hollow microneedle array by two approaches, although the shape of the microneedles array fabricated were similar, the first approach has higher failing due to the blockage of the holes by PDMS residue. It is likely caused during laser cutting process, the third layer PDMS fused with the top-layer PDMS inside holes, which makes the residue left in the holes hard to be removed. Therefore, we choose the second approach as the final fabrication process.

We first evaluated the capability of UV/Ozone-treated microneedles to wick liquids by inserting the tips of the microneedles into a droplet of red dye solution. As shown in Fig. 2-9A, the dyed solution is readily drawn into the microneedles tips upon initial contact and conveyed through the shafts of the needles via surface tension. On average, the time required for the liquid to fill the microneedle was ~30 seconds. Next, we applied the skin patch to an artificial skin model consisting of red dye solution sandwiched between agar gel and Parafilm. As shown in Fig. 2-9B, the solution is quickly extracted by the microneedle array and subsequently transported through the lateral flow immunoassay via surface tension. The time required for liquid to be extracted and wicked through the entire test strip is ~60 seconds.
2.5.4 \( P/HRP2 \) Detection Sensitivity and Specificity

We assessed the sensitivity (i.e. lower limit of detection) of the \( P/HRP2 \) lateral flow immunoassay using dermal interstitial fluid simulant spiked with varying concentrations of recombinant \( P/HRP2 \) antigen from 0 – 1024 ng/mL. We first evaluated the sensitivity of the assay by simply applying a droplet of saline solution (flushing agent) to the sample
region after sample collection. As shown in Fig. 2-10, the intensity of the test line is correlated with the \( PfHRP2 \) concentration which can be detected down to 16 ng/mL.

![Figure 2-10 Assay sensitivity without gold enhancement treatment.](image)

We also investigated the use of a gold enhancement solution to enhance the detection sensitivity. As shown in Fig. 2-11A, the intensity of the test lines is noticeable darker and remain consistent with the \( PfHRP2 \) concentration where the lowest detectable concentration that can be observed by the naked eye is 8 ng/mL. All of the measurements generated a dark control line, validating the test results. The signal amplification process simply entails the application of a droplet of gold enhancement solution onto the test and control lines, and can be easily incorporated in the testing protocol with minimal added effort by the operator. Next, we evaluated the selectivity of the assay by performing measurements of interstitial fluid spiked with \( PfHRP2 \), Pan-\textit{Plasmodium} aldolase or \( PfLDH \), which are other common biomarkers for \textit{Plasmodium falciparum} infection. As shown in Fig. 2-11B, only the \( PfHRP2 \)-containing samples generated “positive” test results, whereas the samples containing the irrelevant proteins generated “negative” results. These results show that our patch offers a similar detection sensitivity as commercial malaria RDTs (7 – 28 ng/mL) [47] and does not cross-react with other common \textit{Plasmodium} protein biomarkers.
Figure 2-11 Sensitivity and specificity of the PfHRP2 immunoassay. (A) Test results of interstitial fluid with increasing concentrations of PfHRP2. (B) Test results of interstitial fluid with 1,024 ng/mL of PfHRP2, PfLDH or Pan-Plasmodium aldolase.

2.5.5 Detection Sensitivity Enhancement Using Gold Enhancement Solution

Several studies were performed to elucidate the mechanism by which gold enhancement treatment of AuNP-IgG conjugates results in improved detection sensitivity of our colloid gold-based immunoassay. We first characterized the morphology of AuNPs-IgG conjugates immobilized on the test line of lateral flow test strips treated with or without gold enhancement solution using secondary electron (SE) and backscattered electron (BSE) imaging. SE and BSE images of the test strip without AuNP-IgG conjugates were also obtained and used as a blank control (Figs. 2-12A and D, respectively). As shown in Fig. 2-12B and E, untreated AuNPs are ~30 nm in diameter (consistent with the manufacturer
specifications), whereas AuNPs treated with gold enhancement solution are enlarged by 50-100% having diameters ranging from 45-60 nm (Figs. 2-12C and F).

Figure 2-12 Scanning electron micrographs of nitrocellulose paper obtained by SE imaging (A) and BSE imaging (D). Scale bars, 1 µm. Scanning electron micrographs of nitrocellulose paper coated with AuNP-IgG conjugates obtained by SE imaging (B) and BSE imaging (E). Scale bars, 100 nm. Scanning electron micrographs of nitrocellulose paper coated with AuNP-IgG conjugates with gold enhancement obtained by SE imaging (C) and BSE imaging (F). Scale bars, 100 nm.

Absorbance spectra of AuNP-IgG conjugate solution treated with and without gold enhancement solution were also obtained, and showed a shift in the absorbance peak from 530 nm (without gold enhancement) to 562 nm (Fig. 2-13A). The shift in the absorbance peak to the lower frequency and higher wavelength is consistent with the change in color of the test and control lines from red to purple (Figs. 2-10 and 2-11, respectively) [48]. Lastly, optical transmittance spectra were obtained from nitrocellulose membrane samples
containing AuNP-IgG conjugates treated with or without gold enhancement solution. As shown in Fig. 2-13B, samples treated with gold enhancement solution exhibited ~6% less light transmittance compared with untreated samples, thereby resulting in ~6% more light reflectance. Therefore, with the same concentration of AuNP-IgG conjugates immobilized on the test line, conjugates with larger-sized AuNPs can generate more light scattering, effectively enhancing (i.e. darkening) the contrast of the test line [49], and enabling lower concentrations of antigen to be detected.

Figure 2-13 Absorbance vs. wavelength and Transmission vs. wavelength.
2.5.6 Proof-of-Concept Demonstration

To mimic the performance of the skin patch *in vivo*, we tested it on young pig cadavers. Cadavers for positive control measurements were dermally-injected with 1,024 ng/mL of *Pf*HRP2, while cadavers for negative control measurements were injected with PBS. The patch was applied to a shaven section of skin pre-injected with *Pf*HRP2 or PBS. On the cadaver injected with *Pf*HRP2, two red lines can clearly be observed in the readout window of the patch, indicating a “positive” result (Fig. 2-14A). In contrast, only the control line is observable in the result window of the patch applied to the cadaver injected with PBS, indicating a “negative” result (Fig. 2-14B). This proof-of-concept test validates the functionality of our skin patch to detect *Pf*HRP2 *in vivo*.

Figure 2-14 Proof-of-concept demonstration of the skin patch. Test results obtained from young pig cadavers dermally-injected with (A) 1,024 ng/mL of PfHRP2 or (B) PBS.
2.6 Discussion and Summary

For most infectious diseases, laboratory-based diagnostic tests exist but suffer from several limitations which hinders their ability to be broadly employed in developing countries. For example, current methods for malaria diagnosis (thick and thin blood smears, ELISA, PCR and RDTs) rely on trained medical personnel and blood sampling, making them poorly suited for mass screening, particularly in communities with blood taboos and large populations of asymptomatic infections. To address this challenge, this skin patch employs a hollow microneedle array for minimally invasive, blood-free sampling of dermal interstitial fluid. Proteomic analysis of dermal interstitial fluid has demonstrated nearly identical protein diversity compared with blood serum and plasma [50], [51], making it a promising source of disease biomarkers. In particular, it has been shown that PfHRP2 can be detected in dermal interstitial fluid of live mice tail-vein-injected with PfHRP2 [22], suggesting that PfHRP2 is also present in dermal interstitial fluid of individuals with Plasmodium falciparum infection. Mean values for PfHRP2 levels in plasma of individuals with malaria infection range from 20 ng/mL – 1,750 ng/mL [52]. Based on prior studies which showed that levels of clinically-relevant proteins (interleukins, C-reactive protein, histidine-rich glycoprotein, etc.) in dermal interstitial fluid and blood are comparable [53], we expect similar concentrations of PfHRP2 between dermal interstitial fluid and blood. Therefore, the sensitivity of this patch (8 ng/mL) makes it useful for detecting varying severities of malaria disease, including asymptomatic infection, uncomplicated malaria and severe malaria. While this study focuses on the detection of PfHRP2 due to its importance for diagnosing malaria infection, we envision that this patch can be used for the detection of other clinically relevant protein biomarkers which have already been identified and
characterized in dermal interstitial fluid [53], thereby further expanding the utility of this skin patch for disease detection and screening.

Much of the existing work on minimally invasive transdermal biosensing employ solid microneedles due to the difficulty in fabricating hollow microneedle arrays. However, the use of solid microneedles for protein capture involves laborious sample processing to recover, concentrate and analyze biomarkers, which complicates the overall testing process. Therefore, we have developed a unique fabrication process to generate hollow microneedle arrays from polymerized SU-8, which are subsequently treated with UV/Ozone to make the surfaces hydrophilic. Based on the hydrophilic nature of the microneedles, dermal interstitial fluid is autonomously extracted upon skin penetration and transported through the lateral flow test strip via surface tension, eliminating the need for human involvement for sample collection. By doing so, the testing procedure for this device is simpler than conventional RDTs making it less dependent on trained medical personnel and enabling more widespread use, particularly in the developing world. By combining the simplicity, speed and low cost of lateral flow technology with the bloodless nature of microneedle sampling, this skin patch has the potential to be a viable diagnostic tool for disease detection and screening in remote and resource-limited settings.
3.1 Motivation

Lateral flow immunochromatographic assays (LFIAs) are one of the simplest and most widely used biosensors for analytical detection. Compared with other types of immunoassays (enzyme-linked immunosorbent assay (ELISA), Western Blot), LIFAs are portable, inexpensive, provide rapid results (< 20 minutes), and do not require complicated sample processing or the use of equipment. Furthermore, LIFAs are compatible with a wide variety of biological matrices, including saliva[54], urine[55], semen[56], serum[57], [58] and whole blood[58], [59]. For these reasons, LIFAs are commonly used for point-of-care diagnostic applications, such as home pregnancy testing and disease detection/screening. Many rapid diagnostic tests (RDTs) for infectious diseases, including malaria[15], HIV[60], tuberculosis[61] and influenza[62], are also based on LIFA architecture. In addition to medical testing, LIFAs are also widely used in veterinary medicine[63], [64], environmental monitoring[65] and food safety testing [66] and bio-agent detection[67], [68].

As described in Section 1.2, LFIAs are comprised of several components, including a sample pad, conjugate release pad, membrane, absorbent pad and backing card. Typically, each of these components is made from a different material[11]. Immunochromatographic test strips are assembled by overlapping the sample, conjugate and absorbent pads with the membrane in a sequential fashion, and mounting the entire assembly onto the backing card. Most LFIAs are also enclosed in plastic cassettes to protect the test strip and provide visual indicators on the device to facilitate the testing process. While conventional LFIA architecture provides an effective means for simplified sample processing and rapid protein detection, the use of multiple materials for the different components, and their assembly
increases the time and costs associated with device fabrication. Furthermore, although the different materials can contribute to the specific function, the multilayer structure could encounter the most common difficulties in LFIA fabrication such as the material incompatibility, contact issue between the overlapping elements or the imperfect characteristics of the material. Due to the variation in surface properties, wicking characteristic, shelf-life and storage condition, choosing the best combination of the materials of LFIA for certain application can be time-consuming. [69], [70] The poor releasing of the conjugation material, the uneven wetting caused by surface imperfection and damage may lead to invalid test or non-specific binding.

Figure 3-1 A) Fabrication process of the inkjet-printed microfluidic immunosensing strip.
B) Multianalyte sensing of two immunological parameters (human and mouse IgG) and a chemical parameter (pH). [71]

To simplify the fabrication of lateral flow immunochromatographic devices, Abe et al. reported an inkjet printing method to pattern microfluidic channels and immobilize
capture antibodies on filter paper as shown in Figure 3-1[71]. While this assay can be fabricated on a single piece of paper substrate, the inkjet patterning process requires several chemical treatment steps, and the limited resolution of the printed features hinders the analytical performance. With inspiration from the single layer immunoassay described by Abe et al., and various methods for fabricating paper-based microfluidic devices, we propose a novel single layer LFIA fabrication process without chemical contamination on the paper surface.

3.2 Biochemicals and Reagents

Monoclonal mouse anti-\(Pf\)HRP2 IgG and monoclonal mouse anti-\(Pf\)HRP2 IgM were purchased from ICL (Portland, OR). Polyclonal rabbit anti-mouse IgG H&L was purchased from Abcam (Cambridge, United Kingdom). Recombinant \(Pf\)HRP2, Pan-\textit{Plasmodium} aldolase and \(Pf\)LDH were purchased from CTK Biotech (Poway, CA). PBS, sucrose, Tween-20, BSA, 2-(N-morpholino) ethanesulfonic acid (MES) buffer, chitosan, and glutaraldehyde were purchased from Sigma-Aldrich (St. Louis, MO). StabilBlock\textsuperscript{®} and StabilCoat\textsuperscript{®} immunoassay stabilizers were purchased from Surmodics (Eden Prairie, MN). 40 nm OD10 colloid AuNPs were purchased from Expecon (San Diego, CA). 2 \(\mu\)m 8\% (w/v) sulfate-Latex Beads solution was purchased from Thermo Fisher Scientific (Waltham, MA). Whatman\textsuperscript{TM} 3MM CHR chromatography paper and Fusion 5 membrane were purchased from GE Healthcare (Chicago, IL). Medical grade adhesive tape was purchased from Adhesives Research, Inc. (Glen Rock, PA). Deionized (DI) water was generated using a Smart2Pure water purification system (Barnstead, Van Nuys, CA). Wax was purchased from local vendor.
3.3 Preparation of AuNPs-IgG conjugates

The preparation of conjugate release antibodies follows the same protocol as described in Section 2.3.2, but OD 10 colloid gold solution was used to improve the intensity of lines instead of adding gold enhancement solution. Briefly, 200 μL of 40 nm OD 10 colloid gold solution was prepared at a concentration of $9 \times 10^{11}$ particles/mL. 12 μg of mouse anti-\(P/f\)HRP2 IgG at a stock concentration 9.28 mg/mL was added to the AuNP solution, vortexed for 30 seconds, gently agitated for 30 minutes and incubated statically at room temperature for 20 minutes. 8 mg of bovine serum albumin (BSA) powder was added to the AuNP-IgG conjugate solution, gently agitated for 30 minutes and incubated at room temperature for 20 minutes. The mixture was centrifuged at 6,500 g for 15 minutes, the supernatant was removed, and the precipitate was resuspended in 200 μL of 20% (w/v) sucrose diluted in StabilBlock® Immunoassay Stabilizer with 0.25% (w/v) Tween-20. The AuNP-IgG conjugate solution was stored at 4°C prior to use.

3.4 Materials Selection

Conventional LFIAIs are based on a nitrocellulose membrane, which is naturally hydrophobic. With the surfactants adding between the nitrocellulose powders, the liquid can flow through the gaps between the powder matrixes. The hydrophobicity of the nitrocellulose powder contributes to the binding reaction at the control and test lines; however, it may also cause significant non-specific binding. Additional blocking reagents are required at different components depending on the biological reagents used. Furthermore, adding blocking reagent to the nitrocellulose membrane may cause the membrane to malfunction due to aggregation and separation of the nitrocellulose powder on membrane strip. The aging of the nitrocellulose membrane will also affect the wicking
rate and protein-binding characteristic, which may influence the performance of the LFIA over time.

Fusion 5 membrane is a glass fiber-based material that contains a plastic binder, which exhibits higher mechanical strength compared to traditional glass fiber strips and its physical properties will not change after long storage. Fusion 5 membrane is naturally hydrophilic, and will not bind under normal test condition, eliminating the need for extra blocking. However, due to its non-binding properties, it is not ideal as a reaction buffer. Therefore, the use of latex beads, acidic solution or detection line treatment is necessary to make a valid single layer LFIA card. In the following sections, multiple approaches for control and test line application are presented. [70]

Whatman™ 3MM CHR chromatography paper is popular material for filtration, separation, chromatographic and blotting applications. This type of membrane is made of pure cellulose fiber that is produced from cotton linters. It can easily bind with proteins without additional treatment, similar with nitrocellulose membrane. Compare to nitrocellulose membrane, cellulose membrane has better durability and is easier to handle. However, it requires a larger amount of liquids for transportation of protein and has a lower protein adsorption rate, therefore, it requires a higher concentration of the antibodies on test and control lines.
3.4.1 Fabrication of the LFIA Card for Substrate Selection

![Fabrication Diagram](image)

Figure 3-2 First Design of Single layer LIFA card for substrate selection.

The initial design of the LIFA card for substrate selection is shown in Fig. 3-2. Each card was made of selected membrane attached to medical grade transparent adhesive, and was cut by a CO$_2$ laser cutter (Universal Laser System) with the patterns designed using AutoCAD software (Autodesk). Antibody solutions for test and control lines were applied using a fine tip (size 10/0) paint brush. A solution of 5 $\mu$L AuNP-antibodies was applied to conjugate release region. Molten candle wax was applied on the bottom edge of the membrane card to prevent the leakage of sample solution to the outer area of the LFIA card.

3.4.2 Fusion 5 Membrane

3.4.2.1 Approach 1 – Preparation of Latex Beads-Conjugated IgG and IgM Antibodies

Since Fusion 5 membrane is designed to not bind with proteins, the immobilization of the control and test line antibodies was challenging. One approach we explored which was suggested by Dr. Kevin Jones from GE Healthcare was to use carrier beads conjugated
with the control and test line antibodies, which would become stuck inside the fiber matrix when applied onto Fusion 5 membrane. The effect of particle size versus line width and intensity on Fusion 5 membrane is shown in Fig. 3-3 [70]. Based on these results, latex beads with particles sizes between 2-3 microns are optimal choices for maintaining the detection sensitivity as well as minimizing the line width.

Figure 3-3 Effect of particles size with a) line width and b) line intensity on Fusion 5 membrane.[70]

To conjugate antibodies with latex beads by passive adsorptions, two solutions of 12.5 μL of 2 μm 8% (w/v) sulfate-LBs solution were diluted with two 200 μL 50mM MES solutions (pH 6.0). The diluted solutions were centrifuged at 6,500 g for 10 minutes at room temperature, the supernatants were removed, and the precipitates were resuspended in two 100 μL 50nM MES buffers to make 1% (w/v) LB solutions while the impurities in the original LBs solutions were washed away. One of the 1% (w/v) LBs solution was added to 20 μL rabbit anti-mouse IgG H&L at a stock concentration of 1 mg/mL, the other LBs solution was added to 23.4 μL mouse anti-PfHRP2 IgM at a stock concentration of 8.53
mg/mL, and gently vortexed both mixtures for 2 hours at room temperature. The mixtures were centrifuged at 6,500 g for 20 minutes, the supernatants were removed, and the precipitates were resuspended in 100 μL of 20 % StabilBlock® Immunoassay Stabilizer in PBS. The IgG-LB and IgM-LB solutions were stored at 4°C overnight prior to use.

![Image of test lines](image)

Figure 3-4 Sensitivity test for detection of PfHRP2 spiked in PBS from 0 - 1,024 ng/mL by applying latex beads conjugated antibodies on control and test lines.

We tested the sensitivity of the LFIA card with latex bead-conjugated antibodies on control and test lines using PBS spiked with varying concentrations of PfHRP2 antigen from 0 – 1,024 ng/mL as shown in Fig. 3-4. The test lines are not visible for the entire group, but control lines are visible.

### 3.4.2.2 Approach 2 – Preparation of IgG and IgM Antibodies in Acidic Buffer

Fusion 5 membrane has a negative charge, therefore, if the antibodies have positive charges, they can be directly dispensed onto the surface of the Fusion 5 membrane and bind by passive adsorption. By diluting the antibody solution in acidic buffer, such as 100mM MES buffer (pH 3.0-3.5), the majority of the antibodies will become positively charged. 1 mg/mL Rabbit anti-mouse IgG H&L and 8.53 mg/mL mouse anti-PfHRP2 IgM will be diluted in 100 mM MES buffer to 200 μg/mL and 800 μg/mL correspondingly.
Figure 3-5 Sensitivity test for detection of PfHRP2 spiked in PBS from 0 - 1,024 ng/mL by applying antibodies in acidic buffer on control and test lines.

We tested the sensitivity of the LFIA card with antibodies in acidic buffer on control and test lines using PBS spiked with varying concentrations of PfHRP2 antigen from 0 – 1,024 ng/mL as shown in Fig. 3-5. The test lines are not visible for the entire group, but control lines are visible with darker red color compared to the results shown in Fig. 3-4.

3.4.2.3 Approach 3 – Treatment for Control and Test Lines on Membrane

Aggregation of monoclonal antibodies is very common due to their physical instability. Aggregation will cause the loss of activity of proteins and may reduce the ability to bind with the carrier beads. [72] Therefore, treating the control and test line region can be carried out enabling the antibodies to directly bind on the Fusion 5 membrane. Dr. Fang Zeng’s group at the University of Jinan used a chitosan-glutaraldehyde cross linker to immobilize capture antibodies on a cellulose paper substrate [73], which was applied to our design. Instead of treating the entire surface of the Fusion 5 membrane, only small amounts of chitosan and glutaraldehyde were applied to the line regions. The cross-linker
solution concentration should be approximately ten times higher than the antibody concentration.

Three droplets of 0.5 μL of 8.0 mg/mL chitosan in 0.2% acetic acid solution were applied at each line region, and dried at room temperature. Three droplets of 1.0 μL 0.4% glutaraldehyde in 0.01M PBS solution were applied to activate the chitosan for 2-3 hrs. Deionized water was used to wash the excess chemicals, and membrane was dried at room temperature. Three droplets of 0.5 μL of 200 μg/mL rabbit anti-mouse IgG H&L and 800 μg/mL mouse anti-PfHRP2 IgM were applied onto control and test lines. Treatment cards were incubated at room temperature and followed by deionized water washing to remove the excess antibodies. Blocking buffer was used to block the unbinding site and excess blockers was washed by deionized water after incubation.

![Image](image.png)

Figure 3-6 Sensitivity test for detection of PfHRP2 spiked in PBS from 0 – 1,024 ng/mL by applying treatment on control and test lines region.

We tested the sensitivity of the LFIA card with chitosan-glutaraldehyde treatment on control and test lines using PBS spiked with varying concentrations of PfHRP2 antigen from 0 – 1,024 ng/mL as shown in Fig. 3-6. Both control and test lines are not visible for
the entire group. During the testing, the treated areas cannot be easily wet and the reagent solution was likely to pass the area from above instead of flow inside the membrane.

3.4.3 Cellulose Chromatography Paper

Due to the challenges encountered with the Fusion 5 membrane, we explored the use of cellulose chromatography paper which offers enhanced adsorption ability with protein, allowing the test and control line antibodies to be directly applied to the paper. However, blocking treatment for the conjugate release region was necessary to prevent the non-specific binding between the AuNPs conjugates and paper. A similar blocking treatment for glass fiber conjugate release pad described in Section 2.3.3 was used. After multiple tries, we used modified solutions with 20% (w/v) Sucrose, 2% (w/v) BSA and 0.25% (w/v) Tween-20 to treat the conjugate release region of the LFIA card, and 15 µL of blocking solution was applied to the test strip followed by drying at 37°C for 15 minutes. This process was repeated for another three times to achieve better blocking performance. Solutions of 200 μg/mL rabbit anti-mouse IgG H&L (Abcam) and 800 μg/mL of mouse anti-PfHRP2 IgM (ICL, Inc.) were drawn using a fine tip (size 10/0) paint brush to generate the control and test lines, respectively.

Figure 3-7 Sensitivity test for detection of PfHRP2 spiked in PBS from 0 - 4,096 ng/mL for chromatography paper-based assay cards.
We tested the sensitivity of the chromatographic paper-based LFIA card using PBS spiked with varying concentrations of \( Pf \) HRP2 antigen from 0 – 4,096 ng/mL as shown in Fig. 3-7. The color intensity of the test line shows positive correlation with the changes in concentration of the \( Pf \) HRP2 antigen. Furthermore, we noticed that larger absorbent pads are essential for using the chromatographic paper and increasing the concentration of the antibody solutions for both test and control lines can contribute to increase the line intensity. Based on these collected results, Whatman™ 3MM CHR cellulose chromatography paper was selected as the substrate material for our single layer lateral flow immunochromatographic assay card.

3.5 Optimization for Design of the Single Layer LFIA Card

3.5.1 Second Design of the Single Layer LFIA Card

The assay card consists of a sample region, a conjugate release region, test and control lines and an enlarged absorbent region (Fig. 3-8). The sample and conjugate release regions were treated with a blocking solution to prevent nonspecific binding of AuNP-antibody conjugates. AuNP-IgG conjugate solution was dispensed on conjugate release region and dried at 37 °C for 1 hour. Experimental procedures for preparing the AuNP-IgG conjugate solution are described in Section 3.3. Mouse anti-\( Pf \) HRP2 IgM at 4 mg/mL and rabbit anti-mouse H&L IgG at 1 mg/mL were used for the test and control lines, respectively. Lines were drawn on the test strip using a fine tip (size 10/0) paint brush and dried at 37 °C for 1 hour. A hydrophobic wax barrier was added to the bottom of the card to prevent liquid backflow. The card is encapsulated between two pieces of self-adhesive plastic film (Adhesive Research) to protect it from the environment and minimize hazards associated with device handling (Fig. 3-8A). The top cover contains cutouts for the sample
dispensing port, test and control line indicators, and the result window. Images of the immunochromatographic device without and with the top cover are shown in Fig. 3-8 B and C, respectively.

Figure 3-8 (A) Exploded view of the single-layer lateral flow immunochromatographic assay. The assay card is encapsulated between two pieces of self-adhesive plastic film. Images of the immunochromatographic device without (B) and with (C) the top cover.

### 3.5.2 Optimization of Strip Width

We first optimized the width of the test strip by performing measurements of samples containing 32 ng/mL of PfHRP2 in PBS using cards with varying strip widths (2 mm, 3 mm, 4 mm and 5 mm). For each measurement, 45 µL of sample was dispensed onto the sample region, followed by 60 µL of PBS. This testing procedure was used for all assay optimization and characterization studies. Images of the assay cards after measurements were obtained using a Cannon CanoScan 9000F scanner. As shown in Fig. 3-9A, the background color of the strips (which corresponds to the amount of AuNP-IgG conjugate remaining on the paper after PBS flushing) correlates to the width of the strip, where the narrower 2 mm- and 3 mm-wide strips exhibit dark background colors making the test line nearly undetectable. In contrast, the background color of the 4 mm- and 5 mm-wide strips
is significantly lighter where the test line is easily observable. We hypothesize that the narrower test strips generate more background color because the blocking solution acts as a dissolvable barrier, which has been shown to increase the flow resistance and reduce the fluid flow speed in narrow paper strips\(^{19}\). Therefore, the transport of AuNP-IgG conjugates is impeded by the slower fluid flow in the narrow strips, causing a large amount of AuNP conjugates to remain on the strip thereby generating a high background signal. In contrast, the faster fluid flow in the wider strips enable AuNP conjugates to be readily transported to the absorbent region, resulting in a low background signal.

To quantify the background signal, mean gray values were obtained from scanned images of assay cards as described in Khan et al.\(^{20}\). Briefly, average gray values were calculated from RGB (red-green-blue) images using ImageJ software, and RGB gray values were converted to CMY (cyan-magenta-yellow) gray values using the formula: CMY gray value = 255 – RGB gray value. Therefore, gray values reported in this article are CMY gray values, and higher gray values represent a darker color indicating a higher concentration of AuNP conjugates. Mean gray values of the test strips support the results shown in Fig. 3-9A, where the 2 mm- and 3 mm-wide strips exhibit 3–5× higher background signals compared with the 4 mm- and 5 mm-wide strips (Fig. 3-9B). The mean gray values of the test line were also obtained and used to calculate the signal-to-background ratio (SBR), which revealed that the 5 mm-wide strips generated the highest SBR (Fig. 3-9C) of all the test strips, making it the optimal width.
Figure 3-9 (A) Test results of samples containing 32 ng/mL of PfHRP2 in PBS using assay cards with 2 mm-, 3 mm-, 4 mm- or 5 mm-wide test strips. The top cover is removed from the card for improved visualization of the test strip. (B) Mean gray values of the background color for various-width test strips. Each bar represents the mean ± standard deviation (SD) of three measurements. (C) Signal-to-background ratios (SBRs) generated from mean gray values of the test line and background color of the test strip. Each bar represents the mean ± SD of three measurements.

3.5.3 Optimization of Blocking Solution

Next, we optimized the composition of the blocking solution. Solutions containing 20% (w/v) sucrose and 0.25% (w/v) Tween-20 were modified by adding 2% (w/v) BSA in
PBS, 5% (w/v) milk powder in PBS, 1% (w/v) casein in PBS or StabilCoat® immunoassay stabilizer. Test strips treated with these blocking solutions were used for measurements of \( P/j \)HRP2 at 0 ng/mL, 32 ng/mL and 1,024 ng/mL in PBS. As shown in Fig. 3-10A-D, test strips treated with the blocking solution containing 2% (w/v) BSA generated the least amount of background color at all antigen concentrations compared with the other blocking solutions. Mean gray values of the test strips confirm that those treated with the 2% (w/v) BSA blocking solution generated the lowest background signal, whereas strips treated with the other three blocking solutions generated the higher background signal (Fig. 3-10E). SBRs calculated obtained from mean gray values also reveal that test strips treated with the 2% (w/v) BSA blocking solution exhibit the highest SBRs at all antigen concentrations compared with strips treated with the other blocking solutions (Fig. 3-10F).
Figure 3-10 Test results of samples containing 0 ng/mL, 32 ng/mL and 1,024 ng/mL of PfHRP2 in PBS using assay cards treated with blocking solution containing (A) StabilCoat® immunoassay stabilizer, (B) 5% (w/v) milk powder, (C) 1% (w/v) casein or (D) 2% (w/v) BSA. The top cover is removed from the card for improved visualization of the test strip. (E) Mean gray values of the background color of test strips treated with different blocking solutions. Each bar represents the mean ± SD of three measurements. (F) SBRs generated from mean gray values of the test line and background color of the test strips. Each bar represents the mean ± SD of three measurements.
3.5.4 Optimization of Volume of AuNPs-conjugates

Figure 3-11 Test results of samples containing 16 ng/mL (A) or 1,024 ng/mL (B) of PfHRP2 in PBS using assay cards with 3 μL, 5 μL, 8 μL or 10 μL of AuNP-IgG conjugate solution. SBRs obtained from mean gray values of the test line and background color of the test strips at 16 ng/mL (C) and 1,024 ng/mL (D) of PfHRP2. Each bar represents the mean ± SD of three measurements.

The last assay parameter that was optimized was the volume of AuNPs-IgG conjugate solution applied to the conjugate release region. Varying volumes (3 μL, 5 μL, 8 μL or 10 μL) of AuNP-IgG conjugate solution were dispensed on the test strips and used for measurements of PfHRP2 at 16 ng/mL and 1,024 ng/mL. Test strips with higher amounts (> 5 μL) of AuNP-IgG conjugate produced more background color compared with those containing a lower amount (< 8 μL) of AuNP-IgG conjugate, particularity at lower (16 ng/mL) antigen concentrations (Fig. 3-11A). At higher (1,024 ng/mL) antigen concentrations, the intensities of the test lines are dark and comparable for all test strips.
Mean gray values show that the test strips containing 5 µL of AuNP-IgG conjugate solution generated the highest SBR for *Pf*HPR2 detection at 16 ng/mL (Fig. 3-11C) and at 1,024 ng/mL (Fig. 3-11D). Based on these results, 5 µL was selected as the optimal AuNP-IgG conjugate volume, which provides the best analytical performance at both low and high antigen concentrations.

### 3.6 Evaluating *Pf*HRP2 Immunoassay Sensitivity and Specificity

The sensitivity of the immunochromatographic assay was evaluated by measuring samples with increasing concentrations of *Pf*HRP2 from 0 to 1,024 ng/mL in PBS and human plasma. As shown in Fig. 3-12A and Fig. 3-13A, the intensity of the test line is correlated with the *Pf*HRP2 concentration which can be detected down to 16 ng/mL with PBS and 4 ng/mL by the naked eye, correspondingly. Each measurement generated a dark control line validating the test results. Mean gray values of the test line (Fig. 3-12B and Fig. 3-13B) also show a correlation between the test line intensity and the *Pf*HRP2 concentration, where the lowest detectable concentration is 16 ng/mL in PBS and 4 ng/mL in human plasma. The higher sensitivity when tested with human plasma samples may be caused by the higher viscosity of human plasma than PBS, which resulted in longer reaction time for the sample and AuNP-conjugates.
Figure 3-12 Test results of samples containing PfHRP2 from 0 to 1,024 ng/mL in PBS. Mean gray values of the test line are obtained from images in panel A. Each data point represents the mean ± SD of three measurements.
Figure 3-13 Test results of samples containing PfHRP2 from 0 to 1,024 ng/mL in human plasma. Mean gray values of the test line are obtained from images in panel A. Each data point represents the mean ± SD of three measurements.
We evaluated the specificity of this assay by testing samples containing *PfHRP2* and other common biomarkers of *P. falciparum* infection, including Pan-*Plasmodium* aldolase (Pan) and *PfLDH*. Non-spiked PBS and human plasma samples were also tested as blank controls. As shown in Fig.3-14A and Fig. 3-15A, only the *PfHRP2*-containing samples generated dark test and control lines, denoting “positive” test results. In contrast, only the control line was generated for samples containing the irrelevant proteins denoting a “negative” result. These collective results indicate that our immunoassay offers adequate detection sensitivity for many sensing applications as well as excellent specificity. Images of the test results were obtained after 25 minutes using a CanoScan 9000F scanner (Cannon).
Figure 3-14 (A) Test results of samples containing PfHRP2, PfLDH or pan-aldolase in PBS, and non-spiked PBS. (B) Mean gray values of the test line obtained from images in panel A. Each bar represents the mean ± SD of three measurements.
Discussion and Summary

We have developed a lateral flow immunochromatographic assay on a single piece of cellulose paper via laser cutting. This assay offers all of the advantages of lateral flow immunoassay technology while being simpler to assemble, and less expensive to produce. Various assay parameters, including the test strip geometry, blocking solution composition and AuNP conjugate solution volume, were optimized to enhance the detection sensitivity of this single-layer immunochromatographic platform. Device characterization and testing
revealed that this assay exhibits a lower detection limit of 4 ng/mL when testing with human plasma by the naked eye and no cross-reactivity with other *P. falciparum* proteins, which is comparable with existing lateral flow immunochromatographic technology. Furthermore, this analytical platform can be readily adapted to detect other protein biomarkers, making it a promising tool for point-of-care testing.
4. Integration of an Electronic Sensor with our Lateral Flow Assay for Quantitative Protein Measurements
4.1 Motivation

Conventional LFIA only provide qualitative results, such as “positive” or “negative”, which while being low cost and simple to operate, can lack information needed for some diagnostic applications. Quantitative results using LFIA can be achieved by analyzing the intensity of the test line using an optical reader. However, optical measurements will only read the particles present on the surface of membrane [74], and information loss during the image analysis can reduce the detection sensitivity. Furthermore, the nitrocellulose membrane used in conventional LFIA has a limited shelf life, and will start to degrade after long storage [70]. The color change of the background will affect the overall reading of the LFIA; therefore, the sensitivity of the optical reading will also decrease with time.

Alternatively, research integrating electronic sensors with lateral flow assays has been carried out, but requires complicated testing procedures or sensing techniques which limits the benefits of LFIA technology. For example, Dr. Ling Yu’s group demonstrated a label-free lateral flow impedimetric test strip for clenbuterol detection, however, the label-free properties of their strip limits the ability to determine the releasing of the analyte and elimination of the non-specific binding [75].

In this chapter, we present an integrated LFIA platform consisting of a wireless impedance sensor on a single-layer lateral flow immunoassay for rapid, quantitative measurements of protein biomarkers.
4.2 Design of Electronic Sensing Element

4.2.1 Theory of Impedance Sensing

As shown in Fig. 4-1, the impedance sensor consists of cellulose paper coated with capture antibodies which is attached to gold interdigitated electrodes deposited on a PET film. An electric potential is applied to the positive and negative electrodes (fingers of the comb-shape capacitor), which generates an electric field. When a sample containing the target antigen is introduced into the system, it binds with the AuNP-labeled secondary antibodies, and the immunocomplexes bind to the immobilized capture antibodies. The presence of the AuNPs-labeled conjugates interferes with the electric field, causing a change in the sensor impedance. The more AuNPs immobilized on the surface, the electrical conductance is likely to increase causing a reduction in the impedance[76]. By

Figure 4-1 Schematic illustration of the impedance sensing region A) without target antigen and AuNP-labeled antibodies, and B) with antigen and AuNP-labeled antibodies.
monitoring the change in the sensor impedance, the concentration of antigen in the sample can be determined.

4.2.2 Design and Fabrication of Impedance Sensor

As shown in Fig. 4.2A, the design of the impedance sensor consists of a comb-shape electrode connected to a coil antenna. The coil antenna is used for wireless communication between the sensor and an impedance analyzer. Changes in width and length of the comb fingers, gap size between the fingers, and size of antenna can result in changes in the impedance and phase curve corresponding to the frequency sweep. Therefore, optimization of these parameters is necessary to enhance the performance of the sensor making it sensitive enough to generate distinguishable results when varying amount of AuNPs conjugates are immobilized on the sensor.

Figure 4-2 (A) Design of a single impedance sensor, scale bar, 5mm. (B) Mask for fabricating comb fingers. and (C) mask for fabricating the coil antenna and connection between fingers.
A two-step thermal evaporating process was used to fabricate the gold sensors. The blue color patterns of the first and second masks (Fig. 4-2A and B) were cut using a CO$_2$ laser cutter. The first and second masks were used to fabricate fingers of capacitor part and rest of the designed pattern, correspondingly. The second mask was carefully aligned under magnifying lens before the second thermal evaporation process.

4.3 Design and Fabrication of the LFIA

4.3.1 Preparation of AuNPs-IgG conjugates

The preparation of conjugate release antibodies followed the same protocol as described in Section 3.3 using an OD 10 colloid gold solution. Briefly, 200 μL of 40 nm OD 10 colloid gold solution was prepared at a concentration of $9 \times 10^{11}$ particles/mL. 12 μg of mouse anti-$Pf$HRP2 IgG at a stock concentration 9.28 mg/mL was added to the AuNP solution, vortexed for 30 seconds, incubated with gently agitated for 30 minutes and incubated at room temperature for 20 minutes. 8 mg of BSA powder was added to the AuNP-IgG conjugate solution, gently agitated for 30 minutes and incubated at room temperature for 20 minutes. The mixture was centrifuged at 6,500 g for 15 minutes, the supernatant was removed, and the precipitate was resuspended in 200 μL of 20% (w/v) sucrose diluted in StabilBlock® Immunoassay Stabilizer with 0.25% (w/v) Tween-20. The AuNP-IgG conjugate solution was stored at 4°C prior to use.

4.3.2 Preparation of LFIA Layer

A single layer LFIA was cut using a CO$_2$ laser cutter. The sample region is designed as an 8 × 8 mm square and the absorbent region is designed as a 15 × 15 mm square to increase the waste absorbing capacity. The channel width between sample and absorbent regions is 5 mm. 15 μL of 20% (w/v) sucrose, 0.25% (w/v) Tween-20 and 2% (w/v) BSA.
in PBS was applied, dried at 37°C for 15 mins and repeated for another three times to treat the conjugate release region. 5 μL of AuNP conjugates was applied at conjugate release region and 4 mg/mL mouse anti- PfHRP2 IgM antibodies (ICL) was drawn using a fine tip (10/0) paint brush followed by drying at 37°C for 1 hour.

4.4 Impedance Sensor-Integrated LFIA Platform

4.4.1 Device Design and Assembly

Figure 4-3 Exploded view of the integrated LFIA platform depicting the impedance sensor, the colloidal gold-based lateral flow immunoassay and self-adhesive tape.

The device consists of a wireless impedance sensor integrated with a colloidal gold-based single layer lateral flow immunoassay encased within self-adhesive tape (Fig. 4-3). The impedance sensor was fabricated on a flexible PET substrate as described in Section 4.2.2. The single-layer lateral flow immunochromatographic assay consists of a cellulose paper on a transparent self-adhesive backing film. 5μL of anti-PfHRP2 IgG antibodies
labeled with OD10 AuNPs was applied to the blocker-treated region and anti-PfHRP2 IgM antibodies was used for a test line. The control line was removed to avoid interference of the signal with the impedance sensor. The top layer of tape contains cutouts for the buffer port, result window, and test (“T”) line indicator (Fig. 4-3). The comb-shape sensing area of the sensor was aligned with the test line on the LFIA strip and attached to top layer of tape. The bottom layer of tape enabled the device to be fully enclosed. The sample loading procedures is similar with that of the skin patch described in Section 2.5.1. Afterwards, the sensor antenna will be aligned with the antenna on the impedance analyzer to read the impedance and phase signals for protein measurements.

4.4.2 Evaluating Detection Sensitivity

Figure 4-4 Experimental setup for measuring the cutout testing region on impedance sensor with wireless measurement set up.
The sensitivity of this device was evaluated by measuring samples with increasing concentrations of PfHRP2 from 0 to 1,024 ng/mL in PBS. 45 μL of each sample was dispensed on the sample pad, followed by 60 μL of PBS, which served as a flushing agent. After the LFIA strip was dried (~10 minutes), the sensor antenna was aligned with the antenna of the impedance analyzer. Since the impedance can be affected by the environmental conditions, the test line region was cut into a 5 × 5 mm square and placed in the center of the sensing region as shown in Fig. 4-4 to minimize the influence of the AuNPs on the absorbent pad and background signal along the strip. The cutout testing elements were placed at the same location on the sensor to minimize changes in the impedance response. The impedance curve under frequency sweep from 560 – 760 MHz with 2 MHz increments was measured using a HP 4191A RF Impedance Analyzer.

4.5 Results

4.5.1 Fabrication of Impedance Sensors

![Image](A) Thermal evaporated gold impedance sensors. (B) First step mask with its holder and (C) second step mask for thermal evaporation process.

Impedance sensors were successfully fabricated by thermal evaporation with a 70 nm gold layer pattern for each step with a finger width of 500 μm, finger length of 7.6 mm,
finger gap of 200 μm and coil antenna width of 3 mm (Fig. 4-5A). PET film was used for the first mask due to its better performance when cutting small features using a CO\textsubscript{2} laser cutter. To prevent shape changes of the mask during thermal evaporation, an additional patterned polymethyl methacrylate (PMMA) plate was used (Fig. 4-5B). A PMMA plate was used to pattern the second mask to prevent the circular region from falling off during the thermal evaporation process (Fig. 4-5C).

4.5.2 Wireless Quantitative PfHRP2 Measurements

Fig. 4-6A and B presents the impedance and phase change curves of samples containing varying concentrations of PfHRP2. Impedance and phase change curves were obtained by subtracting the background signal from the detection signals obtained from the analyzer. Data processing (i.e. curve smoothing) was carried out using Origin software (OriginLab Corporation) by Savitzky-Golay method [77]. The processed impedance and phase change curves are shown in Fig. 4-7A and B, respectively. Although the frequency of the peak values for both impedance and phase shift curves are quite stable for different environmental conditions, the peak values are always fluctuating, even with minor changes in the experimental setup. Therefore, data from all groups were obtained at the same experimental settings. As shown in Fig. 4-7, the presence of PfHRP2-IgG-AuNPs conjugates on the test line region results in a significance change in the impedance signal. Furthermore, the detection signals correlate the with the PfHRP2 concentration, where samples with higher protein concentrations generate lower peak impedance and phase shift measurements. Based on these results, the lowest concentration that could be detected was 8 ng/mL. While these preliminary results demonstrate proof of principle of our prototype
device, further work is needed to optimize the sensor, characterize its analytical performance and evaluate its functionality using clinical samples.

Figure 4-6 (A) Impedance vs. frequency and (B) phase change vs. frequency curves for PBS samples containing varying concentrations of PfHRP2.
Figure 4-7 (A) Impedance vs. frequency and (B) phase change vs. frequency curves after signal processing for samples spiked with varying concentrations of PfHRP2.

4.6 Discussion and Summary

We have presented a unique LFIA platform integrating a wireless impedance sensor on a single-layer lateral flow device for quantitative protein measurements. This assay
offers the advantages of being simpler to assemble, less expensive to fabricate, and can provide quantitative results offering enhanced diagnostic information. Various impedance sensor geometries were evaluated to obtain sharper impedance peaks for enhanced detection sensitivity. Using our prototype device, we generated preliminary data demonstrating quantitative measurements of *PjHRP2* in PBS samples, which could be detected down to 8 ng/mL.
5. Conclusions and Future Work
5.1 Summary of achievements and contributions

In this dissertation, we have presented several unique LFIA technologies aimed at overcoming some of the limitations associated with conventional LFIAs. We demonstrate for the first time a hollow microneedle-based lateral flow immunoassay for colorimetric protein detection in dermal interstitial fluid. Various studies were performed to demonstrate the skin penetration performance and liquid extraction capability of the hollow microneedle array. A simple gold enhancement treatment was employed to enhance the detection sensitivity of this colloidal gold-based lateral flow assay, which could achieve a lower limit of detection of 8 ng/mL. We also elucidate the underlying mechanism of this enhancement mechanism through experimental investigation, which is the first time this is reported in literature. The minimally invasive nature of this skin patch makes it promising for disease testing and screening, particularly in low resource settings.

We also have demonstrated a unique fabrication process to create a LFIA on a single piece of cellulose paper via laser cutting. In contrast to conventional LFIAs that consist of multiple components (sample pad, conjugate pad, membrane, absorbent pad, backing card) and materials, this single-layer LFIA enables simpler and faster fabrication while minimizing material consumption and overall device costs. While offering similar speed, ease-of-use and analytical performance as conventional LFIAs, this single-layer assay can make LFIA development more accessible to researchers in academia and industry.

Lastly, we demonstrated a LFIA platform integrating a wireless impedance sensor on a single-layer lateral flow device for quantitative protein measurements. This device offers the simplicity and low cost of our single-layer LFIA platform with enabling
quantitative protein measurements, which can provide enhanced diagnostic information. The development of this integrated LFIA platform can facilitate the development of other lateral flow assays employing electronic sensors, which can further enhance the usefulness of LFIA technology for point-of-care diagnostic testing.

5.2 Future Work

In Chapter 2, we have successfully fabricated a hollow microneedle-based skin patch for in vivo protein detection. Experiments demonstrated that our hollow MN array can safely penetrate skin and be used for autonomous dermal interstitial fluid extraction via capillary flow. However, the current fabrication process is time-consuming and involves multiple steps (soft baking, post baking, developing etc.). Alterative techniques (e.g. 3D printing) for fabricating hollow MN arrays can be explored to simplify device manufacturing and lower overall costs. Furthermore, the concentration of PfHRP2 and other protein biomarkers may be lower in ISF than in plasma or blood, which may be lower than the sensitivity of our current skin patch. Therefore, additional work to improve the sensitivity of the skin patch can be explored to make this platform useful for diagnostic applications requiring higher sensitivity measurements.

Based on the single layer LFIA developed in Chapter 3, we have developed a new LFIA platform for quantitative protein measurements which is simpler to fabricate than conventional LFIA s. Due to time constraints, only preliminary results demonstrating proof of concept were obtained. Additional studies are needed to optimize the sensor, characterize its analytical performance and evaluate its functionality using clinical samples.
Figure 5-1 Exploded view of the integrated skin patch depicting the microneedle array, the colloidal gold-based lateral flow immunoassay and bandage tape.

Furthermore, we envision that our microneedle-based skin patch (Chapter 2) and integrated LFIA platform (Chapter 4) can be combined to develop a microneedle-based skin patch capable of wireless quantitative protein measurements. The device would consist of a hollow polymeric microneedle array integrated with a colloidal gold-based single layer lateral flow immunoassay and wireless impedance sensor encased within self-adhesive tape, as shown in Fig. 5-1.
BIBLIOGRAPHY
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86


