A lateral flow immunoassay with self-sufficient microfluidic system for enhanced detection of thyroid-stimulating hormone

Santosh Kumar Bikkarolla1*, Sara E McNamee1, Stuart McGregor2, Paul Vance2, Helen McGhee2, Emma L Marlow1, James McLaughlin1*

1School of Engineering, Engineering Research Institute, University of Ulster, Newtownabbey BT37 0QB, U.K.
2Randox Laboratories Ltd, 55 Diamond Road, Crumlin, County Antrim, BT29 4QY, U.K.

*Correspondence: s.k.bikkarolla@ulster.ac.uk; jad.mclaughlin@ulster.ac.uk

Abstract: We report a self-sufficient microfluidic paper-based lateral flow immunoassay device (µLFD) for highly sensitive detection of thyroid-stimulating hormone (TSH). The fabrication of the paper microchannels involves engraving the nitrocellulose (NC) membrane with a CO₂ laser to create narrow flow paths, which constrain the fluid flow over the test zone. The proposed microchannel modified devices were studied for the detection of TSH using gold nanoparticles as labels. The effect of such microchannel modified LFD has led to an improvement in sensitivity by 9 times and limit of detection by 6.6 times due to the slow flow rate of the sample compared with the traditional LFD. Also, the binding of gold nanoparticles over the test line is more uniform in the case of the µLFD, thus minimizing leading-edge effects, resulting in more accurate quantitative analysis. The proposed strategy offers great potential for multiplex detection of biomarkers with increased sensitivity without introducing any hydrophobic materials to the LFD.

Keywords: point-of-care diagnostics; lateral flow immunoassay; paper microfluidics; CO₂ laser fabrication; thyroid-stimulating hormone
1. Introduction

Lateral flow immunoassay devices (LFDs) are suitable for rapid detection of analytes from a finger prick blood sample in low resource settings, are inexpensive, self-powered, and require a small volume of the blood sample. The World Health Organization (WHO) recognizes the importance of point of care (POC) devices such as LFDs in diagnosing diseases and has established criteria for evaluating such devices. Given the acronym “ASSURED,” the criteria specify that devices should be ‘affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free and deliverable to end users’. Current LFDs are simple wicking-based devices, which use gold nanoparticles (AuNPs) for the detection of biomarkers. However, extensive use of LFDs is limited due to a lack of sensitivities, reproducibility, and quantitative analysis issues. One of the main reasons for the lack of sensitivity is that the most common observable labels such as AuNPs, are not particularly sensitive. Labels such as carbon nanomaterials\textsuperscript{1-5}, latex beads\textsuperscript{1}, fluorescent europium nanoparticles\textsuperscript{6}, chemiluminescent labels\textsuperscript{7}, horseradish peroxidase-conjugated AuNPs\textsuperscript{8}, graphene oxide coated with AuNPs\textsuperscript{9}, silver nanoparticles\textsuperscript{10}, and platinum nanoparticles\textsuperscript{11-13} have all been used to improve the performance of LFDs. There are several self-sufficient paper-based devices that have been reported, which improve the performance of the lateral flow assay in terms of reducing non-specific binding, improving quality of test lines, and limit of detection\textsuperscript{14-16}. Zhang et al have developed a self-contained microfluidic lateral flow assay device, which enables the delivery of the reagent and sample through capillary action from two separate flow paths to reduce non-specific binding of the conjugate particles to the NC membrane. Also, to avoid the uneven flow due to the 2D microfluidic network, a liquid impermeable membrane was introduced to mix the reagent and sample flows uniformly across the test strip and resulted in even test lines with good quantification capability\textsuperscript{17}. Wang et al have developed a self-powered microfluidic device that incorporates filter paper for separation of plasma from whole blood and absorbent pad to induce capillary flow in the channels for rapid multiplexed detection of biomarkers from whole blood\textsuperscript{18}.

One of the critical factors that determine the performance of the assay is the immunoreaction time between the antigen and the capture antibody, thus slower migration time of the sample over the test line leads to increased sensitivity. Recent reports have shown that by decreasing the sample flow rate
or by stopping the flow temporarily on the membrane by using novel techniques such as wax printing, PDMS deposition, and use of tunable delay shunts on NC membrane, has led to the improvement of LFD performance. Rivas et al. have fabricated small diameter wax barriers at the starting of the NC membrane to slow the sample flow, which resulted in improvement of the limit of detection (LOD) by 2.6 folds when compared with unmodified NC membrane. Katis et al. constrained fluid flow in a 1 mm wide channel and improved sensitivity by 62 times and LOD by 30 times when compared with unmodified LFD. Simple modifications to the shapes of the NC membrane, conjugate pad, and sample pad have also shown improvement in LFD performance. Zadehkafi et al have shown LFDs with trapezoidal shape NC membrane having narrow width at the absorbent pad and position of the test line far from the conjugation pad has shown improved performance by decreasing LOD from 10 mIU/ml to 5 mIU/ml and test line intensities increased by 2 times for human chorionic gonadotropin biomarker when compared with normal LFDs.

In this study a self-sufficient microfluidic paper-based lateral flow immunoassay device was developed for sensitive detection of TSH. The fabricated narrow flow paths can constrain same amount of fluid flow over the smaller test zone resulting in enhanced detection and removal of leading-edge binding effect. The narrow flow paths also act as self-contained resistors, which decrease the fluid flow rate and increase the immuno reaction time at the test line. Therefore, the narrow flow paths act as a self-sufficient microfluidic system for enhanced detection of TSH. The fabrication process involves engraving with a CO₂ laser to burn the NC membrane across its thickness according to the predesigned pattern. The process of forming narrow flow paths is rapid, a typical NC membrane of 2.5 cm in width and 35 cm long can be engraved within 24 min to produce 55 devices. The process does not require any additional hydrophobic materials, such as wax or photo-polymers, which can result either in adsorption of proteins or contamination of capture antibodies and decreases LFD performance. The process of fabricating paper microchannels is highly reproducible and suitable for the bulk manufacturing process, enabling patterning techniques to be implemented in LFD applications.

To show the advantages of such paper microchannels, we have implemented TSH as a model assay for a proof of principle study to demonstrate the feasibility of the modified µLFDs. TSH is essential in maintaining the body’s metabolism and energetics. TSH plays a key role in developing physical and
mental growth. It is critical for nervous, skeletal, and cardiovascular systems as well as regulating body temperature, heartbeat, and cholesterol. If a thyroid-related disease is untreated several symptoms such as excess gain or loss of weight, cold intolerance, increased heartbeat, infertility, developing low bone density and a range of autoimmune diseases can be observed. TSH levels are normally measured either with the finger prick blood samples or blood obtained through vein. The normal TSH concentration for healthy individuals is in the range of 0.5 to 5 µIU/ml. In the case of subclinical hypothyroidism, TSH levels are in the range of 5 to 10 µIU/ml and TSH levels of above 10 µIU/ml are considered as thyroid failure. Here we demonstrate the potential applicability of µLFDs for the detection of TSH levels with 9 times increased sensitivity and 6.6 times improved LOD when compared with traditional LFDs. The LOD by using µLFDs is 7.5 µIU/ml making the new devices useful for detection of TSH in the clinical range. In addition, the µLFD demonstrates the potential feasibility for multiplex detection of various biomarkers.

2. Materials and Methods

2.1. Reagents and materials

Monoclonal mouse anti-TSH antibody (10-2428, Fitzgerald) and monoclonal mouse anti-TSH antibody (10-2426, Fitzgerald) were used as capture and detection antibodies. TSH antigen derived from human pituitary was obtained from NIBSC. TSH calibrators in serum were obtained from Randox laboratories. Nitrocellulose membrane (CN95, unisart) was obtained from Sartorius. Absorbent pad (Ahlstrom munksjo, A238, A222) was purchased from Kenosha Tapes. Backing card (width 80 mm and thickness 0.015") was purchased from DCN. Carboxyl gold nanoparticle (40 nm, 40 OD) was obtained from Expedeon. N-(3-Dimethyaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC), phosphate buffered saline (PBS), 2-(N-morpholino) ethanesulfonic acid (MES), Tris-buffered saline (TBS), sodium phosphate monobasic monohydrate, sodium phosphate dibasic monohydrate, and sucrose were all purchased from Sigma-Aldrich.
2.2. Gold nanoparticles (AuNP) modification with antibodies

TSH detection antibody was purified using an Amicon filter unit to remove any amine terminated molecules, which can interfere in the conjugation process and the purified antibodies were resuspended in 10 mM potassium phosphate buffer pH 7.4. AuNP-TSH antibody conjugate was prepared by following the recommended protocol from Expeideon. Briefly, AuNPs functionalized with carboxyl group were employed to covalently bind TSH antibodies by using water-soluble EDC. To 50 µl of AuNP suspension (40 OD), 20 µl of 100 µg/ml TSH detection antibodies were mixed followed by addition of 20 µl of 1 mM EDC. The resulting solution was incubated for 30 min at room temperature. Then 1 ml of TBS (containing 0.05% Tween) was added to the mixture and centrifuged at 6200 rpm and 10 °C for 10 min. The supernatant was carefully removed, and the pellet of AuNP-antibody was resuspended in 90 µl of 1XTBS, 0.5 % BSA and 0.05 % Tween to obtain 20 OD for the conjugate.

2.3. Fabrication of LFD and µLFDs

LFDs were prepared in three different configurations: 1) traditional LFD of width 5 mm, 2) single-channel µLFD, and 3) multi-channel µLFD consisting of three parallel channels as shown in Figure 1b. Fabrication of µLFDs consists of two important steps as shown in Figure 1(a): 1) dispensing capture antibodies on the NC membrane. For this step, capture antibodies were diluted in 10 mM phosphate buffer containing 1% of sucrose to obtain a concentration of 1 mg/ml. Anti TSH antibody (10-2428) was dispensed onto NC membrane using a BioDot (ZX1010) dispensing platform at a flow rate of 1 µl/cm to obtain a test line width of 1 mm. Then the NC membrane was dried in an oven at 37 °C for 30 min. 2) Laser irradiation on NC membrane was performed by Universal Laser Systems, VLS2.30 equipped with a wavelength of 10.6 µm pulsed CO2 laser system (25 W). A laser duty cycle of 7.5 %, the scan rate of 20 cm/s and an image density of 1000 ppi were used to fabricate paper microfluidic channels. After the fabrication process, the NC membrane was laminated onto a plastic backing card followed by the absorbent pad with 3 mm overlap on the NC membrane. We simplified the design of the LFD by eliminating the sample pad and conjugate pad. Finally, strips were cut 5 mm wide and stored in aluminum foil bags with desiccant to absorb moisture.
2.4. The lateral flow assay procedure

TSH antigen solutions of different concentrations ranging from 1000 µIU/ml to 5 µIU/ml were prepared in 10 mM PBS, pH 8.0 buffer. To prepare the reaction solution, 5 µl of the Au conjugate (5 OD) was mixed with 45 µl of reaction buffer (10 mM PBS, 1 % BSA and 0.05 % Tween) and 10 µl of TSH antigen solution. Next 60 µl of reaction solution is added to each well of a 96-well plate. LFD was immersed into the sample well and allowed to react until all the solution in the well diffused to the absorbent pad. After the formation of the test line, LFD were dried and scanned by using the HP8600 scanner with 1200 dpi resolution and intensity of the test lines was deduced from ImageJ software. LOD was defined as the minimum concentration that is required to obtain mean intensity that was higher by 3 times of the standard deviation of the blank, without antigen, over the mean intensity of the blank. Each set of experiments were repeated three times in order to check the reproducibility of results.

![Diagram](image.png)

**Figure 1.** (a) The fabrication process of µLFDs. (b) Three different formats of LFD: traditional LFD of 5mm width, multi-channel µLFD consisting of three parallel channels and single channel µLFD.

3. Results and discussion

A number of antibodies for TSH were sourced from different suppliers for suitability in a LFD. Different configurations (n=19) in regards to the capture and detection antibody were evaluated for the
initial antibody screening and characterisation process. Initial experiments focused on spotting antibodies onto the NC membrane and assessing for sensitivity and non-specific binding. From this initial evaluation antibodies from Fitzgerald looked the most promising and were used for the remaining of the study.

To evaluate the stability of the AuNP- TSH conjugate, UV-VIS spectra was collected before and after the conjugation process to measure the optical density (OD) of the conjugate. Figure 2 shows a successful conjugation on gold nanoparticles where the resonance peak is shifted to the right by about 4 nm (red shifted) due to the effect of conjugated protein on the plasmon resonance peak of the gold nanoparticle. Also, the FWHM and OD of the conjugate are the same as that of the gold nanoparticles indicating the conjugate consists of monodispersed gold nanoparticles attached with the detector antibody.

To enhance the analytical sensitivity of LFDs for TSH detection, a new µLFD was developed to decrease the fluid flow rate without adding any additional components and maintaining the traditional format. Fabrication of paper microchannels by using CO$_2$ was performed on capture antibodies deposited NC membrane as shown in Figure 1a to obtain uniform width of the test line. It was observed that dispensing antibodies using the BioDot on a patterned NC membrane resulted in uneven test line shape due to the presence of the narrow flowpaths therefore antibodies were dispensed first. Due to the small spot size (70 µm) and the pulsed mode operation of the CO$_2$ laser, antibodies did not burn during the engraving process of the NC membrane. By following this procedure, paper microchannels of width 0.5 mm were fabricated without damaging the antibodies. To fabricate the paper microchannels, the NC membrane next to the channels was completely etched to make the fluid flow constrained to the designed pathways, as shown in Figure 1b.
Figures 2. Shows the UV-VIS spectra of 40 nm gold nanoparticles before and after conjugation and the insert shows the TEM image of the 40 nm gold nanoparticles.

LFDs were prepared in three different configurations: 1) traditional LFD of width 5 mm, 2) single-channel µLFD, and 3) multi-channel µLFD consisting of three parallel channels as shown in Figure 1b. Single-channel and multi-channel devices were fabricated with channel widths of 0.5 mm and 1 mm. The TSH assay was evaluated on all the strips with varying concentrations as shown in Figure 3 and the intensities of the test line were evaluated using ImageJ. The color test lines were selected and the average intensity values were measured. These values were subtracted from the background noise, which was measured by taking the average of the intensity values before and after the test lines for each individual strip.

First, the performance of the LFDs consisting of absorbent pads A222 and A238 were studied for the TSH assay. Absorbent pads A222 and A238 have wicking rates of 20 s/4cm and 150 s/4cm. LFD laminated with A222 is referred to as the traditional LFD. The LFD laminated with A222 showed LOD of 50 µIU/ml, while using an LFD laminated with A238 a LOD of 20 µIU/ml was obtained. This shows that using a lower wicking rate absorbent pad results in 2.5 times improvement in LOD and an increase in test line intensities. In addition, leading-edge binding is observed more prominently on the LFD laminated with the A222 absorbent pad due to the high wicking rate.

To further improve the performance of the LFD, the NC membrane was patterned in the form of microchannels. For the µLFDs, absorbent pad A238 was used. For analyte concentration of 1000 µIU/ml, the test line intensity increased by 3.5 times on 0.5 mm width constriction when compared with
traditional LFD. It was observed that for lower concentrations, from 250 µIU/ml to 50 µIU/ml, the test line intensities were 9 times higher on 0.5 mm channel constriction when compared with traditional LFD. For this device, the LOD was found to be 7.5 µIU/ml, a 6.6-fold enhancement when compared with the traditional LFD.

In the case of multiplex µLFD of channel width 0.5 mm, the test line intensities are 3 times higher than traditional LFD at an antigen concentration of 1000 µIU/ml. For analyte concentration from 250 µIU/ml to 50 µIU/ml, the test line intensities were 6 times higher when compared with traditional LFD. Multiplex µLFD with 0.5 mm channel width exhibited 7.5 µIU/ml as the LOD, which indicates a 6.6-fold increase in LOD when compared with traditional LFD. The multiplex format of the µLFD is more suitable for detection of multiple biomarkers on one single strip with increased sensitivity when compared with traditional LFD. A detailed comparison of the test line intensities on various formats of LFD was shown in Figure 4a.

**Figure 3.** Scanned images of LFD and µLFD after TSH assay performed at various concentrations ranging from 1000 µIU/ml to 5 µIU/ml. (a) LFD of width 5 mm attached to A222 absorbent pad is considered as traditional LFD. (b) LFD of width 5 mm attached to absorbent A238. (c-d) multiplex channel µLFD of channel width 1 mm and 0.5 mm. (e-f) Single channel µLFD of channel width 1 mm and 0.5 mm. The asterisks (*) indicates LOD.
Figure 4. (a) The average test line intensities at various concentration of TSH on all LFD. (b) Test line intensity profiles on all devices at 1000 µIU/ml concentration of TSH.

Figure 5. (a) Shows the brightness of test line on 1 mm multiplex devices attached A222 and A238 absorbent pads. (b) The assay time or wicking time for 60 µl of the sample on various formats of LFD.

In the case of traditional LFD, the test line intensities were not uniformly distributed i.e., higher intensities were present at the beginning of the test line and weaker intensities were present at the end of the test line, while uniform binding of AuNPs over the test line was formed for µLFD due to the narrow flow paths, as shown in Figure 4b. It can be observed that binding of the AuNPs is more uniform in the case of 0.5 mm µLFD. Thus leading edge binding is minimized in the case of µLFDs, which can result in an accurate quantitative analysis of biomarkers by using lateral flow assay readers.

In the case of multiplex µLFD, absorbent pads (A222 and A238) haven’t shown any difference on the brightness of the test line as shown in the Figure 5a. In addition for multiplex µLFD, the brightness of the test lines were same when measured in serum samples as shown in Figure S1. This indicates that
in the case of µLFD, only patterning significantly lowers the flowrate such that effect of absorbent pad or viscosity of the samples is nullified. To investigate the effect of constriction width in the multiplex devices, µLFDs were fabricated with four channels where the width of alternating channels is 1 mm and 0.5 mm as shown in Figure S2. It has been observed that the brightness of the testline is same on both 1 mm and 0.5 mm channels as shown Figure S3. In addition the brightness of the testline is same as in the case of the device consisting of three channels of 1 mm width. This indicates that the brightness of the test line depends on the total width of the channels but not on the width of single channel in the device.

For all the formats of LFD, wicking time for 60 µl of the sample solution is shown in Figure 5b. It can be observed from Figure 5b that 0.5 mm channel provides a slow flow rate and LFD consisting of absorbent pad A222 provides a faster flow rate. Accordingly, a decrease in LOD, an increase in sensitivity and uniform binding of AuNPs were observed on the LFD providing slower fluid flow rate and consisting of a smaller test zone.

4. Conclusions

We have presented a novel strategy for improving sensitivity and LOD by patterning the NC membrane with a CO₂ laser to constrain fluid flow. These paper microchannels decrease the test area zone and fluid flow rate, thereby increasing reaction time between antigen and capture antibody, leading to the effective formation of the immunocomplex over the test region. Different designs were studied and paper microchannels of width 0.5 mm exhibited improvement in sensitivity by 9 times and LOD by 6.6 times when compared with the traditional LFD. The enhancement of LOD up to 7.5 µIU/ml can make the µLFDs suitable for detection of TSH levels in the clinical domain. This approach of creating narrow constrictions is simpler, less time consuming, does not require any additional materials to create hydrophobic barriers and does not need any alterations to the configuration of LFD when compared with wax printed devices. The proposed method can extend the scope of LFD with patterned techniques to enable its use in a broad range of applications. This study has shown some very promising data and considerations that are worthy of further research to determine its suitability for a commercial diagnostic
Further research will be carried out towards multiplex detection of biomarkers including control lines by using serum samples with a complete device geometry.

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**SUPPLEMENTARY MATERIAL:** See supplementary material for experimental data related with serum and constriction width experiments.

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**DATA AVAILABILITY:** The data that support the findings of this study are available from the corresponding author upon reasonable request.

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