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A Paper Based Lateral Flow Device with Fluid Actuated Valves to Detect Sepsis

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ABSTRACT

This study focuses on the development of a new paper based microfluidic device for the detection of Inter alpha Inhibitor Proteins (IαIP). The key development is on the application of fluidic actuated valves embedded in the paper that allow for sequential manipulation of the sample fluid and multiple reagents in a controlled manner to perform an ELISA protocol on an autonomous paper-based platform. Recent studies proved that the concentration of IαIP is related to the mortality rate of patients who suffer from sepsis. The developed device is able to determine the concentration of IαIP where a low concentration is an indication of sepsis. The microfluidic device consists of paper on which the channel geometries are printed with wax ink. Different layers of the device are stacked on top of one another and held together with double sided tape. After applying the reagents, the device produces the results autonomously in the form of a colored dot. The intensity of the dot is correlated to the concentration of the target analyte IαIP. After optimizing the reagents with regards to reproducibility and optimal signal to noise ratio and sensitivity, a standard curve in the range of 1600-10 ng/ml is produced that links the intensity of color dot to the concentration of IαIP. The limit of detection was estimated to be 25 ng ml -1 which is comparable with the results obtained by conventional ELISA.

Keywords: *microfluidics, ELISA, IαIP, portable device.*

1. INTRODUCTION

The aim of this investigation is to perform a feasibility study of autonomous quantitative detection of a biomarker of Sepsis using a new lateral flow paper based microfluidic valve technology. The international sepsis forum listed the six most common infections that could lead to sepsis as pneumonia, blood-stream infections, intra-abdominal infections, urological infection and surgical wound infection [1]. The evolution from infection into a septic shock occurs quickly (within a few hours), thus, early detection and characterization is vital in reducing mortality. There is, therefore a need for a device that may rapidly detect sepsis or its precursors, since in many cases the symptoms for sepsis are similar to those of the flu [2].

Previous paper-based devices [3] were unable to allow for sequential manipulation of the sample fluid and multiple reagents in a controlled manner to perform ELISA. Commonly, the sequential manipulation is performed by varying the channel lengths which, therefore, increases or decreases fluid flow rates creating a delay if needed. This delay time is limited by the size of the used chip. Apilux et al. performed a sandwich ELISA on nitrocellulose with human chorionic gonadotropin as analyte using solvent ink with acrylic polymer to form barriers. This procedure allows for the conduction of a sandwich ELISA with only one user interaction at the beginning [4]. Fu et al. introduced a two-dimensional paper network (2DPN) as well. The sequential flow of the different reagents (BSA-biotin and gold enhancement solution) was realized by using different channel lengths. Colloidal gold labeled capture species were used to enhance the colored signal and thus improving the limit of detection [5].

In comparison to these aforementioned paper-based devices, Chen et al. remedied this situation by introducing fluid actuated valves imbedded in paper. The use of multiple valves in one device makes it possible for fluids to flow sequentially over a test spot without human intervention. These fluid actuated valves eliminate the need for larger delay channels to control the flow of multiple fluids. This is achieved by progressing the design of the paper-based channel from a 2D to a 3D-geometry. The increased complexity of the device allows for more intricate control over fluid flow rates [6]. Gerbers et al. improved the valve technology by combining the hydrophobic discs into one single layer of paper and replaced surfactant discs with a paste which is spread underneath hydrophobic areas. With these improvements, a chip-yield of 92% was achieved. Using this proprietary technology, it was possible to perform an ELISA on paper by creating a three-fluid four-valve device. They were able to detect Rabbit IgG with a limit of detection (LOD) of 4.8 fm [7]. In this study, an ELISA is performed to detect the concentration of IαIPs in buffer using a device with a similar architecture to that of Gerbers et al. [7].

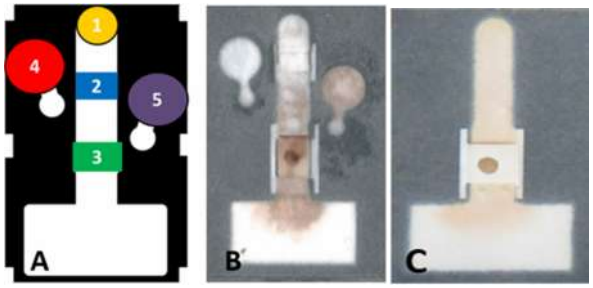

Figure 1. Schematic ELISA Chip (A) shows each component: Sample Pad (#1), Conjugate Pad (#2), Nitrocellulose Membrane (#3), HRP Reservoir (#4) and DAB Reservoir (#5). A fully developed ELISA-Chip (B) is shown after one use along with a Development Chip (C) which was used to optimize biological components.

The results were compared with the ELISA protocol conducted by Lim et al. on a 96- well plate. Also, the results were represented in the form of a dose-response curve which correlates the intensity of a colored dot on the detection area to the concentration of IαIPs in a buffer solution [8].

2. MATERIALS AND METHODS

2.1. Device Fabrication. The proposed chip is assembled with multiple layers of alternating paper- and double-sided tape each containing various elements. This fabrication is described in detail within the following section, however, a schematic drawing of the top view of the chip is shown in Figure 1A along with a result showing the test spot after ELISA was performed in Figure 1B. Figure 1C shows a protocol development chip displaying the test results by a dark spot.

2.1.1. Channel fabrication. The current channel geometry is designed using the vector drawing program Corel Draw® and is printed on a 8x10" filter paper (Grade 41, 20m) from Whatman® with wax ink (Xerox® Genuine Solid Ink Black) using a solid ink printer (Xerox® ColorQube 8570). The paper sheets were cut then with a CO_2 laser cutter (Epilog® Mini 24) to maintain accuracy, precision, and proper alignment during assembly. To make sure wax channels penetrate the entire thickness of each paper sheet, the sheets were placed in a vacuum oven (Isotemp® Model 280A, Fisher Scientific) for 30s-40s at a temperature of 130C – 140 C.

In order to conduct biological experiments on paper, the hydrophilic channels of the chip needed to be blocked so as to prevent non-specific binding of biological materials to the fibers of the paper. Therefore, the blocking of every channel is achieved by fully wetting the paper with a solution of Phosphate-Buffered Saline combined with Tween20 (PBS-T). This is made by mixing Tween20 with Phosphate-Buffered Saline (PBS) to a 0.05% concentration and a 5 wt.% of milk powder. All reagents are manufactured by Sigma Aldrich.

Each devices contain four layers of paper with wax channels: a top layer on which the reagents are stored and applied, a second layer containing the hydrophobic circular regions that form the valves, a third layer containing the delay channels and the regions infused with Tween20 surfactant and the bottom layer consisting of blotting paper (Whatman® Grade GB003 Blotting paper 20x20cm by .8mm thick), which serves to absorb all the fluids and acts as a capillary pump.

2.1.2. Hydrophobic Layer fabrication. The hydrophobic layer is first fabricated according to the procedure described in section 2.1.1 but is not blocked with the BSA-T solution. Following this, one layer is treated by applying a hydrophobic solution which is made from a 5% vol. solution of Acros Organics® Allyltrichlorosilane in Acros Organics® Perfluro-compound (FC-72). Each hydrophobic spot is treated with 8µl (4x2µl) of this hydrophobic solution and is allowed to dry for 24 hours.

2.1.3. Surfactant Paste fabrication. The surfactant discs from Gerbers et. al. are substituted with a surfactant paste which is spread into cutouts in the double-sided tape layer and is then placed underneath the hydrophobic areas. The surfactant paste is made by mixing 0.5g cellulose powder (Fisher Scientific® Cellulose Powder) with 1ml of surfactant solution which is prepared by mixing 35 wt.% of Tween 20 with Ethanol. This solution is then diluted with ultra-pure water to achieve a ratio of 0.25ml/ml water to solution.

2.1.4. Conjugate Pad fabrication. The conjugate pad is fabricated using glass fiber (SterliTech GA-55 Membrane Filters). This pad is where the detection antibodies are placed; however, it must be blocked with 10µl Superblocker® and dried at 37°C on hot plate. Afterwards 10µl of the 20wt.% Sugar Solution (Trehalose and sucrose from Fisher Scientific) is applied onto conjugate pad and is again dried at 37°C on a hot plate. It is then ready for application of the biotin labeled R21 detection antibodies provided by ProThera Biologics, Providence, RI

2.1.5. Nitrocellulose membrane fabrication. The nitrocellulose membrane is cut from a piece of Whatman® 12µm Nitrocellulose 47mm Diameter (G3514143). Afterwards a single drop of 1.5mg/ml of the capture antibody 69.26 (ProThera Biologics, Providence, RI) is pipetted onto the nitrocellulose and allowed to dry.

2.1.6. Assay Development. The ELISA protocol is shown schematically in Figure 2.

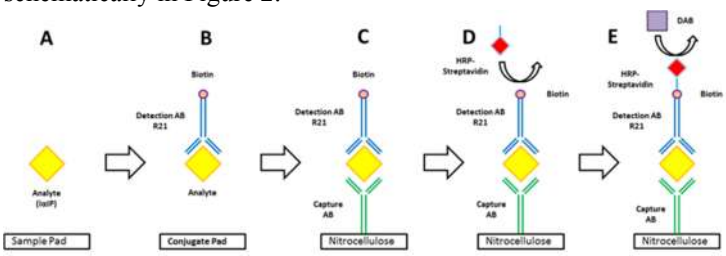

Figure 2. ELISA procedure in lateral flow design: A) analyte (IαIP) is applied to sample pad B) analyte binds to the detection antibody (R21) labeled with biotin (in conjugate pad) C) complex of analyte and detection antibody is captured by capture antibody MAb 69.26 (on nitrocellulose) D) HRP-streptavidin is attached to the biotin E) DAB produces a change in color.

The biological procedure was divided into several steps that were individually tested using the protocol development chip in Figure 1C and then were combined afterwards. The color change from a Diaminobenzidine (DAB) and Horseradish-Peroxidase (HRP) reaction were first tested by mixing both reagents in a small vial and vortexing them for one minute. The color changes from a light brown to a very dark brown color indicating an enzymatic reaction. Next, simple strip tests were performed by preparing a detection area consisting of nitrocellulose membrane on which a drop of detection antibody R21 was applied and allowed to dry. HRP and DAB were then pipetted sequentially onto the sample pad of the protocol development chip and allowed to run down the channel and over the detection area to create a brown colored dot as shown in Figure 1B. Finally, all steps were combined and transferred to the new lateral flow device.

As seen in Figure 3 the developed chip is slightly different compared to the device presented by Gerbers et al. [7]. The

hydrophobic and surfactant discs needed to form each fluidic valve have been replaced by treated sheets of paper.

2.2. Assay Procedure. DAB-Powder (Cat. No. 34001) and Pierce Streptavidin Poly-HRP (Cat. No. 21140) were purchased from Fisher Thermo Scientific. The HRP buffer solution is made by mixing 1.1g of Non-fat Milk Powder in 22g of PBS-T (described in 2.1.1). To produce a liquid DAB substrate from powder, instructions from IHC World are used. The substrate solution has two components: A DAB solution and an H_2O_2 solution. 0.1g of DAB is mixed with 10ml distilled water and 250µl of 10N HCl is added to create the DAB Solution. The solution turns light brown/dark purple. The H2O2 solution is prepared by mixing 100µl of H2O2 in 10ml distilled water. Both solutions are aliquoted into small vials and stored at -20°C if they are not used immediately. Finally, the substrate is produced by mixing 250µl of each reagent in 5ml PBSx1. IαIPs were diluted at various concentrations in blocking buffer which is used in the sample and HRP solutions. The blocking buffer is PBSx1 with 0.1 wt.% milk powder and 0.1 wt.% Tween 20. Varying IαIP dilutions from 1600 ng/ml to10 ng/ml were tested. The IαIP analyte was produced and prepared by ProThera Biologics in Providence, Rhode Island. The negative control is performed using only the blocking buffer. Each dilution was vortexed in advance for at least 90 seconds in order to ensure a homogeneous distribution of the proteins. Note that while in actual blood the maximum concentration for healthy individuals is around 800µg/ml, the maximum concentration in our studies was 1.6 µg/ml. This then would require tests with actual human blood the samples to be diluted by a factor of 1000. The volume for the sample was 80µl, the volume of the wash was 60µl and the volume of the substrate was 120µl. For the assay experiments, first the substrate and the wash were applied to the reservoirs. Then, the sample was applied to the sample pad which starts the test. From these tests, a dose response curve was produced.

In accordance with Figure 1A, the IαIP solution is applied onto the sample pad (#1). It flows over the conjugate pad (#2), where the detection antibody R21 is stored and the conjugate IαIP +R21 is formed. This conjugate then flows over the nitrocellulose membrane (#3) where a drop of 1.5mg/ml of the capture antibody 69.26 has been deposited and dried. The complex MAb 69.26+ IαIP+R21+biotin is formed in the test spot. Meanwhile, a portion of the sample fluid from the sample pad flows via the delay channels towards the valves over which the Horseradish-Peroxidase (HRP) (#4) and 3,3 Diaminobenzidine (DAB) (#5) reagents have been preloaded. The valves are opened in sequence whereby, first, HRP flows over the test spot where it connects via the streptavidin to the biotin on antibody R21. The DAB substrate is triggered and begins to flow next, interacting with the HRP enzymes and resulting in a color change. Trying to imitate a conventional ELISA, each of the described steps was performed according to the ELISA procedure from ProThera Biologics. The detailed flow of the fluid is displayed in Figure 3. It takes around 30s, after the sample fluid was applied onto the sample pad, for the HRP to reach the top main channel. After 300s the full amount of HRP ran over the nitrocellulose membrane. Next, the DAB wicks through and flows over the detection area. The whole ELISA test is completed within 600s.

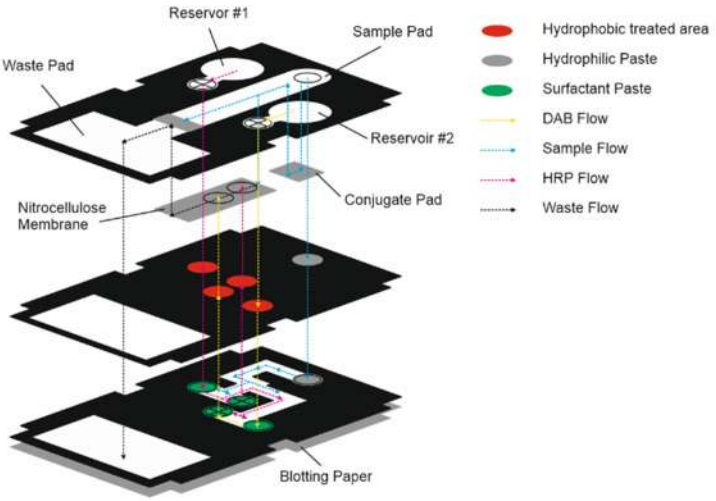

Figure 3. The HRP and DAB solutions are applied onto the reservoir spots 1 and 2 respectively. Then the analyte is pipetted onto the sample pad and runs over the conjugate pad and the nitrocellulose membrane. Meanwhile it wicks through to the bottom layer and triggers the reservoir fluids which then flow sequentially over the test spot on the nitrocellulose membrane.

3. EXPERIMENTAL SECTION

3.1. Optimization of Detection Antibody. The optimization of detection antibody R21 plays an important role regarding background noise and sensitivity of an ELISA. To achieve the best signal/noise ratio, different concentrations of R21 were tested. Therefore, an ELISA is conducted with different dilution factors of R21 in PBS (1, 5, 10, 25, 50, 100 vol.%). Every other reagent involved in that ELISA was kept at a constant concentration. First, the conjugate pad, where the detection antibody is applied, is blocked with 10µl Superblocker® and dried at 37°C on hot plate. The conjugate pad is fabricated using fiberglass paper sheets (SterliTech GA-55 Membrane Filters). Second, 10µl of varying amounts of R21 in 20wt.% Sugar Solution are applied onto conjugate pad and again dried at 37°C on a hot plate. After the full ELISA is conducted, the intensities of signal and background on the nitrocellulose membrane were measured using the software ImageJ®. Six different dilution factors of R21 were tested. In **Figure 4** the proportion of signal to background is plotted over the dilution factors of R21. It can clearly be seen that the optimal dilution factor of R21 is 1:10.

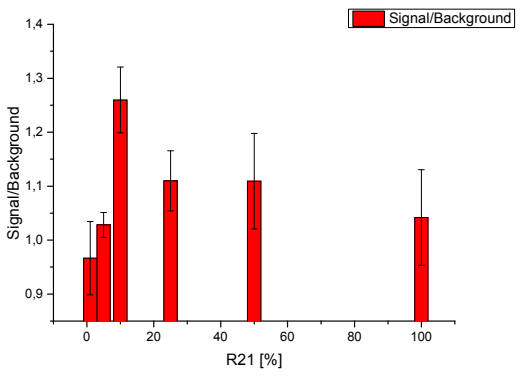

Figure 4. Optimization of R21.

3.2. Optimization of Conjugate Release. In order to improve the release of detection antibody R21 from the conjugate

pad, the concentrations of the two different types of sugar (trehalose and sucrose) were varied according to Fridley et al. who showed that sugar facilitates the rehydration of dried reagents as well as prevents any physical damage [9]. Therefore, the release from a conjugate pad (4mm x 5mm) treated with different sugar concentrations was tested. The size of the conjugate pad was optimized by testing the ability to release bound proteins. The two sizes tested were 4mm x 5mm and 5mm x 5mm. Also, different pad sizes varying materials (with or w/o binder) and blocking approaches (blocked or non-blocked) were conducted as well.

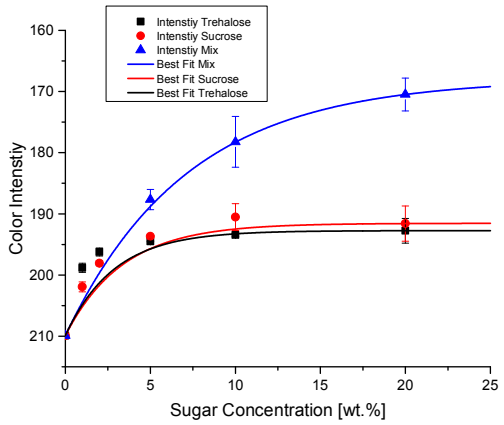

Figure 5. Optimization of Conjugate Release. The red and black curve represent a sugar solution of sucrose and trehalose. The blue curve represents a mix of trehalose and sucrose.

Sucrose and trehalose were dissolved in SuperBlock blocking buffer in five different concentrations (1%, 2%, 5%, 10% and 20%). Furthermore, a mixes of trehalose and sucrose in concentrations of 5%, 10% and 20% were tested. First, the conjugate pad was blocked using 10µl of Non-Fat Milk powder and dried at 37°C on hot plate. The Non-Fat Milk blocker is produced by mixing 1.1g of Non-Fat Milk powder in 22g of PBST. Afterwards, it is treated using 10µl of a sugar solution containing trehalose or sucrose or a mixture in Non-Fat Milk blocker. In the sugar solution, the concentration of R21 is kept constant at 20 vol.% and dried on a hot plate at 37°C. Afterwards, 10µl of HRP with a concentration of 7 µg/ml is applied onto the conjugate pad so that the streptavidin of the HRP can bind to the biotin on the R21. To determine the best release conditions, the HRP-R21 complex is washed out from the conjugate pad onto a first spot on a blotting paper first by a 40µl of PBSx1 solution applied onto the pad. Then, after moving the pad to a second spot on the blotting paper, a 30µl of PBSx1 is applied. Finally, four more additional washing steps are conducted with 20µl of PBSx1. To induce the color change 10µl of DAB are pipetted onto each spot where the R21/HRP conjugate pad is washed out. In Figure 5, the intensities of the colored dot in the detection area are plotted over different wt.% of trehalose, sucrose mixture. In this figure lower intensity values indicated the colored spot is darker or that signal is higher. The graph shows that the color intensities of the washed out conjugate pad are nearly identical and have a saturation point around a color intensity of 195. A mixture of trehalose and sucrose provides the lowest intensity of the colored dot which correlates to the highest amount of detection antibody could be washed out of the conjugate pad.

The curve of the sugar mix can be approximated by Equation (1). At a theoretical value of 100 wt.%, the optimal intensity of the colored spot can be achieved. Since the sugar mix becomes very viscous when using an amount of sugar of 20 wt.% or higher and the improvement of the intensity is only around 1.5%, a concentration of 20 wt. % is used for the experiments.

$$y = 41.98e^{\frac{-x}{7.15}} + 167.92$$

3.3. Optimization of HRP. The optimization of HRP plays an important role with regard to background noise and sensitivity of an ELISA. To achieve the best signal/noise ratio different concentrations of HRP were tested. Therefore, an ELISA is conducted with different concentrations of HRP, which were 0.01, 0.05, 0.1, 1, 5 µg/ml. Every other reagent involved in that ELISA was held at a constant concentration. Five different concentrations of HRP are tested to evaluate the optimal dilution for the ELISA

In Figure 6, the Signal to Background ratio is plotted over the concentration of HRP

It is clear that the optimum concentration of HRP is 1µg/ml which provides the highest signal to background noise ratio.

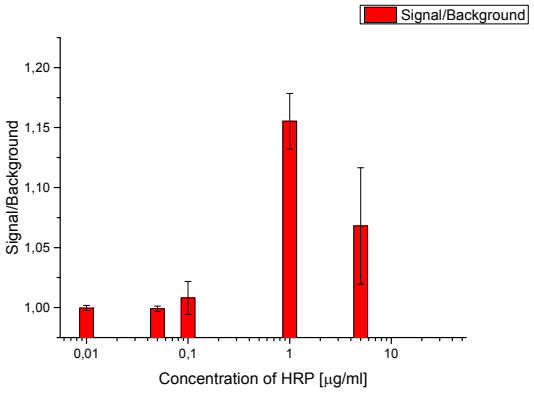

Figure 6. Optimization of HRP.

4. RESULTS SECTION

In Figure 7, the signal-background ratio is plotted over different concentrations of IαIP. As can be seen from this figure, the lower the concentration of IαIP correlates with a lower signal to background ratio. This dose-response curve makes it possible to run a paper based ELISA with IαIP. After estimating the intensity of the detection spot and the background, it is possible to determine the concentration of the analyte. The concentrations which are used to create the standard curve are in a range of 1600 to 10 ng/ml.

Table 2 shows a series of micro photographs after an ELISA was conducted.

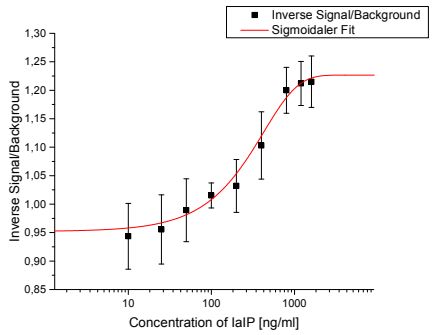

Figure 7. IαIP dose-response curve.

The images show a close-up of the test spot in the nictrocellulose detection pad in the devices. The spots darkness decreases as the concentration of IαIP is decreased from 800 ng/ml to 25 ng/ml. Since at a concentration of 25-100 ng/ml the signal is barely stronger than the background and most of all three signal-to-noise ratios overlap the Limit of Detection was estimated to be 25 ng/ml. The negative control is shown as well.

In the human body the concentration is around 800 to 200 mg/ml. It is worth noting that the concentration of the test analyte is three orders of magnitude lower than the actual concentration of IαIP typically found in human blood which means the test is usable to detect low levels of IαIP in buffered solutions.

Table 1. Detection Areas for the different concentrations of IαIP.

800	400	200	100	50	25	NC
						

5. CONCLUSIONS

The results have shown that it is possible to develop an autonomous and quantitative new paper-based lateral flow device for the detection of IαIP via ELISA. In this work the HRP and DAB fluids were pipetted onto their respective pads. In the final product, these reagents will be stored in cavities in the housing and will be loaded into their respective pads by a simple action by the operator, prior to loading the sample. The loading of the sample stats the assay, because the sample helps turn on the valves beneath the reagents allowing them to flow in a predetermined sequence. The detection limit obtained is comparable with the results from conventional ELISA. The detectable concentration is three orders of magnitude lower than concentration of IαIP in human blood.

The next step would be to further optimize the developed procedure by implementing a washing step as introduced by Giannakos [10]. The final step would be to use human blood as analyte to prove the usability in real world cases.

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