Development of Micro-Lateral Flow Strips Compatible with Micro-Volume Samples for Immunochemical Assays

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ABSTRACT

MicroPlumbers Microsciences LLC has developed elements of a miniaturized lateral flow strip (LFS) based device that is compatible with low-pain microvolume blood extraction methods having sample volumes as small as 1 microliter. A related device design with parallel configuration of miniaturized LFS that performs 10 independent assays from a single drop of sample (approx. 25 microliters) has also been demonstrated. The elements of a miniaturized LFS including application, capture, control and absorber pads were fabricated from backed sheets of nitrocellulose by milling on a computer numeric control table top milling machine.

The functionality of these miniaturized LFSs has been demonstrated using avidin and troponin I antibody modified microspheres and the appropriate ligands immobilized at capture pads of the miniaturized LFS.

Keywords: microfluidics, lateral flow strip, micro-lateral flow strip, micro-volume, painless blood extraction

1. INTRODUCTION

Lateral flow strip tests are immunochromatographic assays that become increasingly popular for many different diagnostic applications. The benefits of immunochromatographic tests include their user-friendly format, very short time to result, long-term stability over a wide range of climates, and relatively low cost of development and manufacturing. The benefits of the lateral flow strip immunochromatographic test format are well known. However, currently available lateral flow strip assays are not compatible with microliter sample volumes, such as volumes generated by low-pain blood extraction methods, nor are they able to test for 10 or more different analytes from a single drop of sample. Here we describe fabrication of miniaturized flow strips capable of processing samples as small as 1 µl and performing 10 independent assays from a 25 µL sample. A prototype individual flow strip is shown in Figure 1.

Conventional LFS are generally composite test strips of approximately 70 mm x 5 mm x 1 mm, containing separation materials for sample preparation, pre-deposited reagents and controls, a wicking layer that drives the sample fluid and the reagents through and along the strip, and a visualization pad that typically shows a control and a sample signal line. Because of their physical size, they still require a minimum of 1-2 drops (25-50 µl) of blood, which makes them incompatible with painless blood extraction devices. These conventional lateral flow strips cannot be easily miniaturized to work with 1 µl sample volumes due to inherent limitations. Test strip dead volumes must be significantly less than the sample size, yet sufficiently-thin nitrocellulose membranes are not available from membrane manufacturers due to strength and consistency limitations. Application and confinement of antibodies and beads to specific areas on the strips during preparation is generally accomplished by applying volumes that are small compared to the strip volume so that they quickly dry, limiting their spread from the point of application. But controlled spreading of these preparation solutions is problematic as miniaturized strip volumes approach solution application volumes. Inability to concentrate the test stripes results in diffuse detection regions making the reduced sample volumes difficult to detect.

Figure 1: *A prototype micro-lateral flow strip. 1 µL sample droplet shown applied to application pad (1), via pipette. Flow proceeds through detection pad (2) and control pad (3) to absorber pad (4).*

2. METHODS

Non-traditional fabrication methods are required to produce functional miniaturized lateral flow strips. A successful implementation must be extremely thin, have a tiny dead volume and, to sufficiently concentrate the available particles, its detection and control stripes must be contained within small areas with sharp well-defined edges. MicroPlumbers Microsciences LLC has developed designs and fabrication methods that meet these goals. We have developed methods to successfully reduce the thickness of existing nitrocellulose membranes, without

adversely affecting their flow and capture characteristics, lowering dead volumes to approximately 0.1 µl. We have also developed methods to limit spreading of membrane preparation treatments to well-defined zones in the nitrocellulose flow strips, within areas as small as 100 µm square. These methods allow us to produce extremely thin (down to 10 µm) nitrocellulose membranes with very small dead volumes, and well-defined conjugate, detection and control zones. In order to achieve these results, mathematical models and experimental results were used to guide prototype design and fabrication.

2.1 Mathematical modeling

Proprietary MicroPlumbers TADS™ multiphysics modeling and simulation software was utilized to evaluate fluid propagation in the porous material (e.g., nitrocellulose) used in the miniaturized LFS. Fluid propagation rate and the interaction of reagents and beads were explored. This was accomplished by using mathematical relations calibrated by experimental data to model the diffusion and advection of the beads through the nitrocellulose membrane to ensure that the locations

(a) After wicking of 0.11 microliters of bead solution

(b) After wicking of 0.59 microliters of bead solution.

Figure 2: *Snapshots at two different times in a simulation by the TADS software of the movement of beads through the prototype strip of Fig. 1. The color key represents the normalized concentration of beads. The regions without nitrocellulose have zero bead concentration (dark blue). The perspective view makes the strip appear shorter than it is.*

containing capture antibodies would be thoroughly exposed to the bead solutions.

The velocity of the wetting front of the solvent was calculated from a balance of capillary and viscous forces as a function of surface tension, pore size and porosity, fluid viscosity, and distance of the wetted front from the liquid source. Once the membrane is fully wetted, the solvent velocity follows Darcy's law and is proportional to the membrane permeability. Since these relations and the membrane characteristics are approximations, the wetting velocity was calibrated by wetting experiments conducted on the Millipore HF240 membrane. The wetting speed is proportional to the square root of the wetting time, allowing determination of the average wetting speed for the length of the prototype strips.

Figure 2 shows color visualizations of the movement of beads through the prototype strip of Fig. 1 at different times in a simulation performed with the TADS software. The color key represents the normalized concentration of beads. Compared to the other components of the membrane, the nitrocellulose bridges between pads are narrower; these regions without nitrocellulose have zero bead concentration (dark blue). The simulations show and experiments confirmed that, even though the test and control pads are wider than the nitrocellulose bridges between them, a sufficiently uniform bead concentration is obtained throughout each test or control pad through a combination of wicking and diffusion processes. All bound antibodies on the pads should be sufficiently exposed to beads.

2.2 Micro-Lateral Flow Strip Fabrication

Miniaturized LFSs were fabricated from nitrocellulose membranes with polyester backing material (Hi-Flow Plus Membrane, Millipore, Bedford, MA.) Machine milling was selected as the method to reduce membrane thickness. This method is reliable for both producing thin nitrocellulose membranes with flow and chemical characteristics similar to commercially available membranes, and for producing well defined capture zones. Milling was accomplished on a computer numeric control table top milling machine (Sherline Products, Vista, CA) using precision chucks (New England Brass & Tool, Winchester MA) and precision milling tools (Drill Technology, Ada, MI). Milling control programs utilizing optimized milling methods were internally developed to successfully shave the nitrocellulose to thicknesses less than 10 µm, or to completely remove it from the polyester backing material. The wetting and flow characteristics of the shaved nitrocellulose were similar to unshaven membranes. To fabricate miniaturized LFSs, nitrocellulose membranes were initially milled to our design thickness of 25 µm, excluding the region reserved for the absorber pads. Then individual strips were created by further patterned complete removal of excess nitrocellulose from the polyester backing. This resulted in 25 µm thick pads comprising application, capture, control and initial portions of the absorber pads, with polyester backing exposed between features. The bulk of the absorber pad
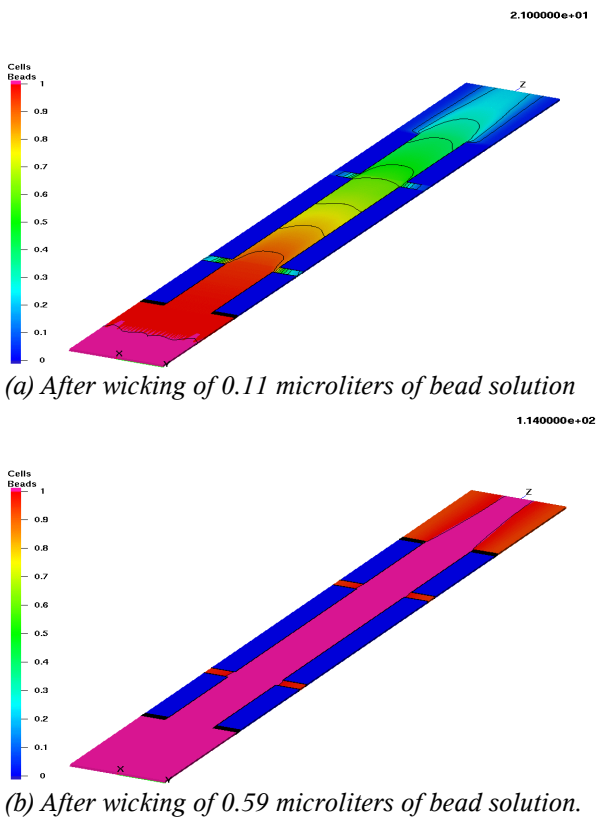
regions were composed of unmilled nitrocellulose to maximize their absorption volume. Figure 3 shows most of the primary components of the micro-lateral flow strip, the application pad (A) acts as a sample receiving mechanism, the detection (B) and control pads (C) act as antibody-based detection and control zones, the absorber pad (D) acts as a fluid absorbing mechanism.
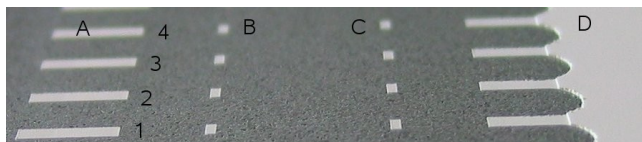

Figure 3: After milling, four 25 µm thick micro flow test strips consisting of (A) application pad, (B) detection pad, (C) control pad, and (D) absorber pad, milled out of polyester backed nitrocellulose membrane. Detection and control pads are 1mm wide x 0.5mm long. Ridge to unmilled portion of the 135 µm thick absorber pad is clearly visible on the right.

To fluidically connect these components together three different transport attachment mechanisms were tested, capillary channels formed by patterned plastic laminates, capillary bridges formed from patterned acrylic, and capillary bridges formed from patterned polyester backed nitrocellulose. We found that all three methods worked well with some differences in transport speed, reusability, ease of fabrication and ease of use. For the final device nitrocellulose transport bridges were selected and fabricated due to their consistent and slower wicking rates, with one placed directly on top of each strip to complete the flow path. The nitrocellulose bridges were milled so that they retained nitrocellulose only in those regions between each primary component of the flow strip. The transport bridge, seen attached in Figure 1, acts as the wicking membrane.

The basic functionality of the micro-lateral flow strip in consistently transporting 1 µL of liquid and bead solutions from application pad, through capture pads to absorber pad was verified successfully.

2.3 Assays Selected for Testing

Two assays were selected for proof of concept, binding of NeutrAvidin labeled beads, and binding of cardiac Troponin I (cTnI). Basic micro-lateral flow strip functionality tests were conducted using NeutrAvidin modified microspheres (FluoSpheres, 0.2 µm Carlsbad, CA) with associated antibodies (Biotinylated Anti-Bovine IgG, KPL, Gaithersburg, MD).

Microspheres for cTnI assay were prepared by attaching of anti-troponin monoclonal antibody (Biospacific, Emeryville, CA) to aldehyde/sulphate latex FluoSpheres® Invitrogen, Eugene, OR) using a protocol suggested by the manufacturer. Excess unbound antibody was removed using dialysis membranes with MWCO of 1,000,000 Da (SpectrumLabs, Rancho Dominguez, CA.). Polyclonal troponin I antibody raised in goat (BiosPacific, Emeryville, CA) was used in test strip capture pads

Cardiac Troponin I (BiosPacific, Emeryville, CA) was used as a model analyte.

3. RESULTS

3.1 Individual Strip Tests

Tests were run utilizing complete micro-lateral flow strips to gauge the effectiveness of bead transport and capture using NeutrAvidin labeled microspheres. Miniaturized LFS with biotin labeled antibodies adsorbed to capture pads were used in this set of experiments. To deposit biotin-IgG on the nitrocellulose capture pads, 1 µL of 0.25 mg/ml antibody solution was applied to each capture pad. After a 15 minute incubation the pads were washed with 5 µL of 2% BSA and with 5 µL of PBS. Application pads and transport bridges were treated with 2% BSA solution and PBS. Capture pads acting as a negative control were also treated with BSA solution and PBS. Bead dispersions were prepared in different concentrations and 1 µL samples were applied to each flow strip, followed by 6 µL of a buffer solution. Following the tests the strips were prepared for fluorescent readings. Capture and control pads were cut and placed in wells on 96-well black plates. Each piece was secured at the bottom of the wells using latex o-rings. PBS was added to each well in the amount of 25 ml. Fluorescence intensity was measured using a fluorescence plate reader (Synergy HT-1 BioTek Instruments, Winooski, VT) at an excitation wavelength of 485 nm and an emission of 528 nm.

Figure 3 shows the results of the fluorescent measurements as a ratio between measurements of specific binding at the capture pad and non-specific binding at the control pad for each measured strip, their ratio predictably declining from the highest applied number (1 µL of 1140×10^6 beads/µL) to approximately 280×10^3 beads where the ratio was 1 (equivalent amount of noise or non-specific binding). For comparison purposes, our calculations suggest the 280×10^3 bead limit corresponds to a lower detection limit of 0.15 ng/mL of cTnI (for a 1 µL sample).

3.2 Multiple Strip (Parallel) Tests

The feasibility of multiplexing a volume of 25 µl into 10 micro-lateral flow assays was also investigated. One set of ten strips was prepared for parallel flow by placing a plastic wicking strip across all its application pads, connecting them together, so any applied sample would immediately wick across. The plastic wicking strip was cut to 1mm width from wet-media film (Dura-Lar, Dick Blick Art Materials, Galesburg, IL). A single 25 µL drop of 2 ng/mL cTnI, pre-mixed with modified beads, was applied to the center of the plastic wicking strip, it was immediately wicked to and wet all 10 application pads. Flow proceeded along all 10 test strips as shown in Figure 4. After most of the sample had been taken up and transported to the absorber pads the sample was washed through by adding two separate applications of 20 µL of buffer. The time for flow to reach the absorber pads was 8

minutes, with buffer follow up the total was 12 minutes. Figure 4 shows the result of parallel flow through the ten test strips from a single applied drop of sample. The wetted boundary clearly shows liquid transport to all ten absorber pads in approximately equivalent volumes. Detection of beads at the absorber and control pads indicated that bead transport through the flow strip functioned as desired, however binding of cTnI did not occur as expected. This could happen for a number of reasons: (1) There is a possibility that antibodies did not bind to the detection pads during the preparation phase. Subsequent washing with BSA would then have effectively blocked those pads from non-specific binding resulting in the observed behavior. (2) If the anti-troponin monoclonal antibodies did not bind during bead preparation, there would be no binding sites on the beads and antigen would not be captured. Regretfully we did not have time to test the cTnI assay further during this period, but expect that further testing and refinement of assay recipies will lead to a successful outcome. Although the detection of cTnI was inconclusive all other aspects of the test, sample application, bead transport, control pad binding, and delivery to the absorber pad, were successful. Since the fluid volume taken up by the connecting wicking strip was very small the resulting sample volume delivered to each strip was more than double the 1 µL design value, pointing to an even greater multiplexing capability of possibly over 20 parallel channels for a 25 µL sample.

4.   CONCLUSIONS

Miniaturized lateral flow strips (LFS) having sample volumes as small as 1 microliter have been demonstrated. A related device design with parallel configuration has also been demonstrated to perform 10 independent assays from a single drop of sample (approx. 25 microliters). CNC machine milling of nitrocellulose has been shown to be a viable method to produce miniaturized lateral flow strips. The basic functionality of these micro-lateral flow strip in consistently transporting 1 µL of liquid and bead solutions from application pad, through capture pads to absorber pad was verified successfully. We were able to verify flow strip functionality and successfully detect beads utilizing NeutrAvidin-Biotin binding using quantitative (fluorescence) means. Although the detection of cTnI was inconclusive we are confident that it was not due to flow strip functionality issues but specific to the assay, since all other aspects of the test were successful. Our lower detection limit was 280x10³ beads using fluorescent readings which, our calculations suggest, corresponds to a lower detection limit of 0.15 ng/mL of cTnI (for a 1 µL sample).
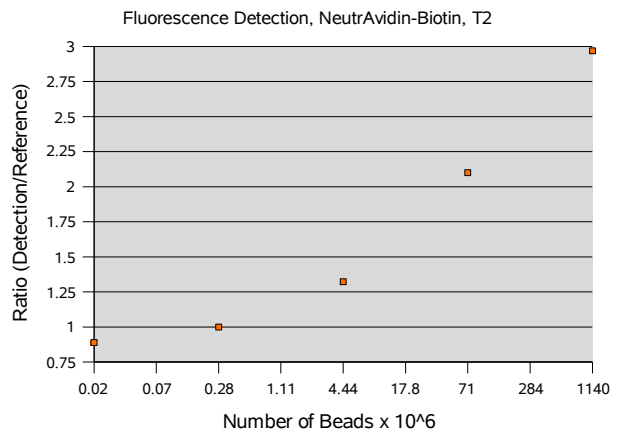

Figure 3: *Ratio of fluorescent readings from detection pads & control pads (used as reference), where detection pads were treated with biotin labeled antibody and control pads were treated with BSA to prevent specific binding. The data indicates detection sensitivity down to 280*x10³ *beads.*
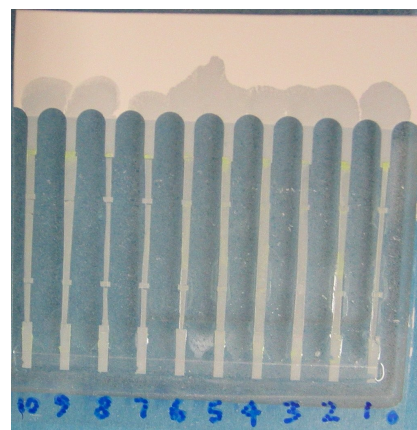

Figure 4: *Multiplexed parallel configuration of 10 independent test strips. One 25 µL drop was applied at center lower edge of the plastic wicking cover, between 6 and 5. The sample was immediately wicked horizontally across all application pads (10 to 1) from where flow through each strip proceeded upwards at the normal rate. The results show approximately even flow distribution of the single 25 µL sample through all 10 test strips.*