Practical High-Performance Lateral Flow Assay Based on Autonomous Microfluidic Replacement on a Film

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Although paper-based microfluidic devices are an ideal platform for point-of-care (POC) diagnostics, it is difficult to achieve microfluidic control required for sensitive analyses such as ELISA on a paper substrate. Here, we present a novel lateral-flow test chip that can perform operations similar to a pump, such as flowing, stopping, and replacing a solution, just by adding the solution onto an inlet port. The chip was fabricated by laminating paper, film, and adhesive tape. For sensitive and accurate detection in an immunoassay, the transparency and flatness of the substrate is crucial for precise analysis of weak light generated by a specific antigen-antibody reaction; however, paper is not flat and is opaque. Therefore, transparent film was applied to the detection area of the chip in this study. The chip showed a good correlation at 0.1 - 100 ng ml⁻¹ concentrations of C-reactive protein, demonstrating high quantitative analysis of CRP in serum suitable for clinical trials. The signal intensity of the novel chip was higher than that of a chip made of nitrocellulose membrane, and the variation was smaller. The limit of detection of the chip was 0.1 ng ml⁻¹, whereas that of the nitrocellulose membrane was 100 ng ml⁻¹. This novel chip can be used for sensitive sandwich immunoassays just by adding solutions.

Keywords Lateral flow, autonomous microfluidic, point-of-care, C-reactive protein, ELISA

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Introduction

Simple ELISA technologies have been used as tools for point-of-care-testing (POCT), and such assays are typically carried out in microtiter plates in the laboratory. Among POCT devices, paper-based devices have been seen as the ideal platform for point-of-care (POC) diagnostics due to their very low cost, simplicity, and inherent ability to wick fluids.1–15 Fluid transport eliminates expensive pumping instrumentation and complicated handling.16,17 The porous structure of paper is very convenient for dot-immunobinding assays, and cellulose itself has high affinity to biomolecules.18,19 On the other hand, these features are not always sufficient to achieve sensitive and accurate sandwich immunoassays. A conventional ELISA is generally performed by measuring the optical density on a transparent substrate via multiple washing and incubation steps. For optical detection, the transparency and flatness of the substrate are very important for precise analysis of weak light generated by the immunoassay; however, paper is non-flat and opaque. Since paper is composed of many cellulose fibers, it is very difficult to accurately detect weak light generated inside and on the cellulose fiber matrix. Also, as compared with a microchannel composed of glass or plastic material, paper fibers tend to have a high degree of nonspecific adsorption, which reduces the sensitivity and accuracy of detection. In addition, the coarse surface of paper negatively affects the analysis of an optical signal.20–24 Such multiple reactions and washing need to be carried out independently of the respective reaction, and contamination needs to be avoided. Conventional immunoassays using 96-well microplates completely replace the solution with every reaction and wash, but complicated steps and know-how are required for paper substrates. Furthermore, 96-well microplates have incubation performed in a stationary phase. On the other hand, practical and prevalent paper-based immunoassays, such as diabetes and pregnancy chromatographic test strips, are performed in flow. The principle behind the reaction and washing of sequential immunoassays is completely different when carried out in stationary phase vs. flow.

Paper-based immunoassays like ELISA are highly prevalent, and novel protocols have been developed for highly selective analysis. To the best of our knowledge, no reports concerning improvements in paper opacity and substrate roughness of paper test chips have been published. In this manuscript, we describe a novel analytical test chip with reduced opacity and roughness by using a transparent film as the substrate. Its fabrication can be accomplished inexpensively by laminating paper, film, and adhesive tape. Since the light is emitted on transparent film rather than opaque paper, small changes in light during emission or fluorescence can be clearly detected. Furthermore, the substrate on which the liquid flows and biochemical reactions occur is a microchannel surface composed of film and tape, not a paper matrix. Therefore, there are no drawbacks like nonspecific adsorption to paper fiber. Furthermore, the novel chip can direct solutions in ways that normally require a pump,

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such as flowing, stopping, and replacing them, just by adding new solutions to the inlet port.

For sandwich immunoassays with the novel chip, reagents and antibody solutions were dropped onto the inlet port of the chip with a dropper. When liquid is dropped onto the inlet port, a certain amount is automatically held in the microchannel, and the surplus liquid is recovered to absorbent paper. Therefore, external equipment such as a micropipettor is unnecessary. As a model sample, we chose C-reactive protein (CRP) for the initial development because it is a strong biomarker for prognosis in chronic heart and kidney disease.

To verify the detection capability of this test chip, we fabricated another analytical test chip using nitrocellulose membrane as the substrate, and compared the results of immunoassays using the same protocol. Sandwich immunoassays were investigated using two different methods, both fluorescence detection using a fluorescein-labeled antibody and chemiluminescence detection using an HRP-labeled antibody. As a result, we found that the analytical test chip made of paper, film, and adhesive tape could achieve sensitive detection as high as 1000-times or more than that of the test chip using a nitrocellulose membrane. These results offer a first step toward breakthrough technology for on-site sensitive and accurate immunoassay-based diagnostics.

**Experimental**

**Reagents**

Anti-human CRP (goat) fluorescein conjugated antibody (F00249) was purchased from Boster (CA, USA). Purified human CRP (CRP15-N-100) and goat anti-human CRP (CRP11-A) were purchased from Alpha Diagnostic Intl. Inc. (San Antonio, TX, USA). HRP-conjugated detection antibody (CY-8071) was employed as the product in a Circulex High-Sensitivity CRP ELISA Kit (Nagano, Japan). Phosphate-buffered saline (PBS, 1 wt%) was used as the dilution buffer for plasma samples, and 0.4 wt% bovine serum albumin (BSA) solution was used for blocking the polyvinylidene chloride (PVDC) film and the nitrocellulose membrane (HF09004X SS, Millipore, USA). Peripheral venous blood samples were collected from healthy subjects by standard venipuncture. All blood samples were collected into sterile centrifuge tubes and the serum was obtained by centrifugation at 8000 rpm for 5 min. The serum was stored at –80°C until use.

**Apparatus**

For antibody immobilization, surface modification of the chip was performed using soft plasma etching devices of SEDE-GE type (Meiwafosis, Japan). Microwave-induced argon plasma is an efficient technique for biomolecule immobilization on a resin surface. In addition, there is a concomitant decrease in the
contact angle of the surface after argon plasma etching. Antibody immobilization to the PVDC film and nitrocellulose membrane was performed using piezoelectric inkjet (Cluster Tech., Japan) printing-based microdeposition of antibody solution after plasma treatment. Piezoelectric inkjet deposition enables picoliter quantities of liquid with drop-on-demand control. The printing head (PulseInjector: Cluster Technology Co., Ltd. Japan) is a piezo-driven drop-on-demand head made from plastic and uses epoxy resin composite as the structural material. The head exhibits excellent chemical resistance similar to plastic and very low biomolecular adsorption compared with glass. Combining the PulseInjector with a dedicated driving unit (WaveBuilderR) allows for ready adjustment of the ejection drive waveform, the ejection rate, and the driving voltage, enabling droplets to be ejected on demand in picoliter quantities.

Fluorescence detection by sandwich immunoassay was performed using fluorescence micro-optical probe heads applied to SELOF micro-optics (FLE1100-02-470/530; Nippon Sheet Glass Co. Ltd., Japan) and its dedicated detector including controller and software (FLE1100; Nippon Sheet Glass Co. Ltd.). Chemiluminescence was measured using a WSE-6100H-CSP LuminoGraph (ATTO, Japan).

Chip structure and assay method

ELISA protocols involve numerous steps of dispensing, incubation, and washing. When applying ELISA to a microfluidic operation, dispensing by pipet is needed to place a precise volume of liquid into the microchannel. The incubation step requires flowing then stopping a precise volume of liquid into the microchannel repeatedly. Washing steps are needed to remove unbound reactants by solution replacement. The analytical test chip in this study performed the above operations just by adding solutions to the inlet port. Hence, no technical skill is required even for a sandwich immunoassay, which consists of multiple incubation and washing steps. In the first step of the assay, the applied sample is moved into the microchannel by lateral flow as the driving force (Fig. 1a). The microchannel is composed of the film and double-faced tape (Fig. 1b) so that the adhesive of the tape does not have interfacial tension. The material cost of the paper is about 1.0 yen for a size of 50 × 50 mm. The cost of the film is about 0.09 yen for 50 × 50 mm, and for the tape is about 1.7 yen for a size of 50 × 50 mm. The cost of these materials for a single chip is about 3 yen. The antigen of the sample solution specifically reacts with the antibody being immobilized on the microchannel surface. Since the specific interfacial area of the sample and the microchannel surface is large, the reaction of antigen and antibody is completed in a short time; the straight microchannel is 1000 μm and approximately 50 μm deep. The depth of the microchannel cannot be defined precisely because the film is a soft material. Next, reagents and washing solution are sequentially dropped onto the inlet. The reaction and bound/free separation are sequentially performed by the addition of reagents and washing. Finally, the detection reagent is dropped and the signal is analyzed.
In principle, an aqueous solution of sample or wash should move without the assistance of capillary force, because a solution flowing by capillary force maintains a strong tendency to stay inside the microchannel. Even if a second solution is dropped at the inlet, solution replacement does not occur. Hence, the solution flow is driven not by capillary force but by lateral flow. The mechanism of the solution replacement in the chip is described in Fig. 2. The solution dropped at the inlet moves into the microchannel and the surplus is absorbed into the absorbent paper, which is placed downstream of the microchannel (Fig. 2a). The surplus solution is absorbed by the absorbent pad, whereby solution equivalent to the microchannel volume remains in the microchannel (Fig. 2b). This means that the solution flow is autonomously divided into two parts, the absorbent pad and the microchannel. Indeed, solution replacement was confirmed even using a visibly thick fluorescence solution and water solution. Figure 2c shows the driving forces when solution replacement occurs. Therefore, the chip can continuously perform multiple reactions just by dropping solution onto the inlet port.

**Results and Discussion**

**Solution replacement**

To confirm the accuracy of the solution replacement, fluorescence intensity was monitored when a fluorescence solution and water solution were dropped one-by-one alternately on the inlet (Fig. 3). The monitoring of the detection area of the chip was performed using a fluorescence micro-optical probe (FLE1100-02-470/530) and detector (FLE1100) (Fig. 3a). As one drop of fluorescent solution was placed, the intensity immediately increased to a constant value (Fig. 3b). The intensity at each time point was almost the same. On the other hand, as one drop of water solution was placed, the intensity decreased immediately to a constant value (Fig. 3b). Likewise, the intensity at each time point was almost the same. Since the fluorescence detection is sensitive, this result shows that reliable data can be collected after solution replacement. We confirmed that solution replacement could be performed at least 10 times.

**Sandwich immunoassay**

Sandwich immunoassays were performed by two different methods, ELISA and immunofluorescence assay. To verify the detection capability of the chip, we fabricated another analytical test chip using nitrocellulose membrane as a substrate, and compared the results of sandwich immunoassays using the same protocol (Fig. 4). PBS solution containing BSA as a blocking compound was placed on the microchannel surface after fixation of the antibody on the surface. The ELISA and immunofluorescence assays were performed by the procedure shown in Figs. 4b and 4c. The incubation time for the reaction was set to 3 min and the washing was set to 1 min. After 3 and 1 min had elapsed, the solution was dropped onto the inlet with a dropper. The results are shown in Figs. 5 and 6. The sample solutions were prepared by diluting CRP in 10-fold diluted serum. The determination of the serum concentration of CRP was performed with a microtitration plate via the conventional method according to the supplied instructions. To make a calibration curve, we diluted CRP with PBS to 0.1, 1.0, 10, and 100 ng ml⁻¹ to be used as samples. Serum was diluted 10-times with PBS, and the serum concentration of CRP was calculated from the standard curve and expressed as ng ml⁻¹.

Three deposits of antibody to capture the antigen were made into each microchannel, and the solutions for sandwich immunoassay flowed into the microchannel, allowing triplicate determination of chemiluminescence intensity and fluorescence intensity obtained by the respective assays. From the chemiluminescence data shown in Figs. 5a and 5b, the novel chip in this study showed good correlation at a range of 0.1 - 100 ng ml⁻¹ of CRP concentration, and the $r^2$ value from a simple linear regression ($y = 1.867\ln(x) + 5.928$) was 0.9816. The signal intensity of the novel chip was higher than that of the nitrocellulose membrane, and the variation was smaller. Also, the nitrocellulose membrane showed no significant difference at
concentrations of 0.1 – 10 ng ml\(^{-1}\) of CRP. Compared with the chemiluminescence intensity shown in Fig. 5, the nitrocellulose membrane chip had a lot of nonspecific adsorption and a small signal-to-noise ratio.

To further the development for on-site POCT, we performed sandwich immunoassays using a fluorescence micro-optical probe (FLE1100-02-470/530) and a small detector (FLE1100) (Fig. 6). This system, composed of the novel chip and fluorescence detection, can measure weak fluorescence signals even in high ambient light just by adding reagents. The results were much the same as the chemiluminescence shown in Fig. 5. The dynamic range was 0.1 – 100 ng ml\(^{-1}\) of CRP concentration, and the limit of detection (LOD) was 0.1 ng ml\(^{-1}\) (Table 1), whereas the LOD of the nitrocellulose membrane was 100 ng ml\(^{-1}\). A strong correlation was also obtained as determined by simple linear regression analysis \(y = 2.5146\ln(x) + 20.98, r^2 = 0.9419\). There was little or no nonspecific adsorption or contamination by solution replacement with the chip. However, the novel chip has some disadvantages compared to the nitrocellulose membrane. In particular, paper devices such as nitrocellulose membranes are darker than the film due to enrichment, promoting visibly clear detection. Western blotting, a relatively complex procedure, can identify tiny amounts of antigen as protein. But to the best of our knowledge, there have been no reports demonstrating that such highly sensitive detection can be accomplished just by adding solution onto an inlet port. Therefore, the performance as seen in Table 1 indicates that this system can have significant impact in areas such as clinical POCT.

**Conclusions**

We fabricated a novel analytical test chip inexpensively by laminating paper, film, and adhesive tape, and the chip successfully simulated operations that would normally require a pump, such as flowing, stopping, and replacing a solution, just by adding the solution onto an inlet port. To overcome the
opacity and roughness of paper, transparent film was used as an alternative. A sandwich immunoassay, which normally needs multiple washing and incubation steps, was realized just by adding the solution. The LOD of the chip was 0.1 ng ml⁻¹, whereas that of a nitrocellulose membrane was 100 ng ml⁻¹. The variation of the chip was also very small. The chip has several fascinating features, such as ease of handling, high signal detection on transparent film, and successful solution replacement without a pump. We believe that this chip has significant advantages for POCT.

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Fig. 6 Quantitative sandwich immunofluorescence assay using the novel chip and nitrocellulose membrane chip. Fluorescence intensities of serum CRP at concentrations of 0.1 - 100 ng ml$^{-1}$ were compared with and without antibody immobilization to evaluate signal-to-noise ratio. (a) Intensity of the novel chip had good correlation at a range of 0.1 - 100 ng ml$^{-1}$. (b) Intensity of the nitrocellulose membrane chip showed no significant difference at 0.1 - 10 ng ml$^{-1}$.

Table 1 Immunoassay performance of a stop-and-flow-based chip made of paper, film, and tape, and comparison to a nitrocellulose membrane

|                    | Novel chip     | Nitrocellulose membrane |
|--------------------|----------------|-------------------------|
| LOD                | 0.1 ng ml$^{-1}$ | 100 ng ml$^{-1}$       |
| Fluorescence       | 0.1 ng ml$^{-1}$ | 100 ng ml$^{-1}$       |
| Sample volume      | 3 - 10 μL      | 3 - 10 μL              |
| Nonspecific adsorption | Little or none | High                |
| Material cost      | Very low (paper, tape, film) | Very low (paper) |
| Contamination after solution replacement | Little or none | High |

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