Wax-Assisted One-Step Enzyme-Linked Immunosorbent Assay on Lateral Flow Test Devices

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Introduction

Lateral flow tests (LFTs) are widely used analytical tools characterized by portability, operator simplicity and short analysis times. A remaining challenge is their limited analytical sensitivity, which in classical immunoassay formats is overcome by enzyme-linked immunosorbent assay (ELISA) formats. The implementation of ELISA to an LFT format however, is hampered by the complexity of the procedure requiring the enzyme substrate addition after sample addition. In this work, a simple method for automation of this procedure without user interference is presented. Originally used sample pads of LFTs have been replaced by hydrophobic wax-modified filter paper-based sample pads to realize a delayed flow a pre-deposited colorimetric ELISA substrate without other alterations to the classical lateral-flow immunoassay format. The performance of the system has been characterized by visualizing flow behavior and final proof-of-concept is provided by a model mouse IgG assay, achieving a limit of detection of 15.8 ng mL–1 from just a single application of the sample solution.

Keywords Lateral flow immunoassay, ELISA, wax printing, sequential reagent delivery, signal amplification

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has been achieved from a single operation step (i.e., sample introduction only), this came at the cost of enlarged dimensions of the nitrocellulose-based device.

This work introduces a one-step SRD approach targeting ELISAs without any need for major design modification from that of classical LFT devices. In the elaborated LFT devices, neither multiple pipetting steps nor extended nitrocellulose flow channels are involved. To achieve this goal, a hydrophobic wax-modified filter paper with pre-deposited enzyme substrate has been employed as the sample pad. The hydrophobic wax barrier prepared through a part of the paper thickness results in formation of a standing sample droplet at the inlet. Gradual rehydration of the pre-deposited enzyme substrate into the sample droplet leads to delayed delivery of this reagent, enabling the ELISA procedure from a single pipetting of sample using the “classical” LFT format. A model experiment using colorants has demonstrated that the occurrence of SRD is achievable only in the presence of the wax barrier on the sample inlet. In addition, one-step ELISA on the LFT device has been successfully performed by using mouse IgG as a model analyte. The achieved limit of detection (LoD) of 15.8 ng mL\(^{-1}\) was comparable to that of a microtiter plate-based ELISA (10 ng mL\(^{-1}\)).

**Experimental**

**Chemicals and instruments**

Mouse IgG (I5381), anti-mouse IgG (M8642) and anti-mouse IgG-alkaline phosphatase (ALP) conjugate (A3562) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nitrocellulose membranes with laminated backing (HF180CM100) and glass fiber conjugate pads (GFDX203000) were purchased from Merck Millipore (Billerica, MA, USA). Absorbent pads (CF7) and Whatman No. 4 filter paper were purchased from GE Healthcare Life Sciences (Buckinghamshire, UK). The BCIP/NBT (5-bromo-4-chloro-3-indolylphosphate p-toluidine salt, nitro-blue tetrazolium chloride) enzyme substrate solution kit was purchased from Nacalai Tesque (Kyoto, Japan). Acid Red 52, Evans Blue, sucrose, bovine serum albumin (BSA), Tween 20, MgCl\(_2\)-6H\(_2\)O, casein, boric acid, Na\(_2\)HPO\(_4\) and NaH\(_2\)PO\(_4\)·2H\(_2\)O were purchased from Wako Pure Chemical Industries (Osaka, Japan). Tris(hydroxymethyl)aminomethane was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

A ColorQube 8570 wax printer (Xerox, Norwalk, CT, USA) was used to pattern the hydrophobic wax barrier onto the filter paper substrate. A QHE325 hot laminator (Meikoshokai Co., Ltd., Tokyo, Japan) was used for the post-print heating of the wax. A DC-200N disc cutter (Carl Jimuki Co., Ltd., Tokyo, Japan) was used to cut the porous substrate materials required for the construction of the LFT device.

**Preparation of the wax-printed sample pad**

Whatman No. 4 filter paper cut into A4 size was fed into the wax printer to obtain the sample pad. The schematic illustrations in Fig. 1 show the dimensions and wax printing conditions of the filter paper-based sample pad. All wax printing was performed in black-and-white printing mode with the corresponding R, G, B color settings shown in Fig. 1. The printed wax was allowed to partially penetrate into the thickness of the filter paper by passing the print-modified sheet through the hot laminator. Finally, 182 sample pads prepared on an A4 sheet were cut into individual sections by using the disc cutter. Then, 2 μL of the BCIP/NBT enzyme substrate solution (10-fold diluted with the substrate buffer solution provided in the kit) was manually pipetted two times with a 15 min interval onto the sample pad (location specified in Fig. 1), followed by drying for 30 min at 40°C.

For the model experiment using dyes, 2 μL of 0.8%(w/w) aqueous Evans Blue solution was deposited in place of the enzyme substrate.

**Preparation of the conjugate pad**

The GFDX203000 glass fiber was first cut into pieces of 5 × 4 mm\(^2\) size. Blocking was performed by depositing 20 μL of Tris solution (50 mM, pH 8.0) containing 5%(w/v) BSA, 0.1%(w/v) Tween 20, and 1 mM MgCl\(_2\). After complete drying, 5 μL of anti-mouse IgG-ALP conjugate (5000-fold diluted by pH 8.0, 50 mM Tris with 1 mM MgCl\(_2\)) was applied.

For the model experiment using dyes, 4 μL of 0.16%(w/w) Acid Red 52 with 8%(w/w) sucrose solution was deposited in place of the ALP-labeled antibody.

**Preparation of the nitrocellulose membrane**

For the preparation of the control and test lines, 100 μL of mouse IgG and anti-mouse IgG solutions (0.5 mg mL\(^{-1}\), in pH 7.4, 10 mM phosphate buffer) were filled into separate Ballsign Souffle Pens (Sakura, Color Products Corp., Osaka, Japan). After loading the respective pen into a Silhouette CAMEO desktop electronic plotter/cutter using a penholder, control and test lines were drawn onto the nitrocellulose membrane at 2 and 2.5 cm distances from the top, respectively. For this purpose, the drawing pattern was designed using the Silhouette Studio software. The detailed instrument settings for line drawing were as follows: media type, copy paper; ratchet blade setting, 1; cutting velocity, 3 cm/s; thickness setting, 1. After complete drying, the nitrocellulose membrane was cut into 4 mm wide strips using the disc cutter. Actual images of the setup of the electronic plotter/cutter and a schematic showing the line drawing positions on nitrocellulose are provided in Fig. S1 of the Supporting Information.

**Preparation of the absorbent pad**

The CF7 absorbent pad (22 mm × 50 m) was cut into pieces of 4 × 22 mm\(^2\) size with the disk cutter and was used without any pretreatment.

**Assembly and use of the lateral flow test (LFT) devices**

The dimension of a single LFT device and the arrangement of
the single functional pads are schematically shown in Fig. S2 (Supporting Information). For the assembly of the LFT devices, the adhesive seal on one side of the nitrocellulose membrane backing card was first peeled off and the absorbent pad was attached with 2 mm overlap on the nitrocellulose membrane. After removing the second adhesive seal, the conjugate pad and the wax-printed sample pad were consecutively attached onto the nitrocellulose membrane with 2.5 mm overlap on the corresponding underlying substrate.

For the model assay, 200 μL of mouse IgG standard solution prepared in a Tris buffer (pH 9.5, 100 mM, containing 5 mM MgCl2) was dropped onto the sample pad. For quantitative signal readout, devices were scanned 60 min after sample application with a 9000F MARKII color scanner (Canon, Tokyo, Japan). After conversion to a 16-bit grayscale image, the mean gray intensity values of the test line (75 × 15 pixel rectangle) was analyzed using the ImageJ color analysis software (NIH, Bethesda, MD, USA).

Results and Discussion

Mechanism of wax-assisted SRD on LFT devices

The function and the requirements of the wax barrier on the sample pad have been first confirmed using Acid Red 52 and Evans Blue dyes. In this model experiment, the enzyme substrate on the sample pad and the enzyme-labeled anti-mouse IgG on the conjugate pad have been replaced by Evans Blue and Acid Red 52, respectively, to visualize the flow characteristics. In the presence of the wax barrier on the sample pad, introduction of 200 μL of water resulted in sequential delivery of the two dyes at the position simulating the test line (Fig. 2a). As seen in the expanded view of the position corresponding to the test line in Fig. 2a, the color of the nitrocellulose strip underwent a transition from colorless to pink and to blue over time, demonstrating the successful sequential delivery of the two components after a single “sample” application to the sample pad of the device. On the other hand, the result in Fig. 2b shows that the device without the wax barrier on the sample pad failed in the sequential delivery of Acid Red 52 and Evans Blue.

The postulated working principle of the wax-assisted SRD system is shown in Fig. 3. The sample pad enabling SRD is composed of the wax pattern surrounding the hydrophilic filter paper region, the thin wax barrier formed through only a part of the paper thickness, and the pre-deposited reagent for delayed delivery (Fig. 3a). In this system, the sample liquid applied to the sample pad forms a standing droplet, due to the flow resistance created by the presence of the thin wax barrier (Fig. 3b, i), preventing the fast bulk liquid flow. Whereas the sample liquid is from the beginning continuously transported in the LFT device via capillary forces, the pre-deposited reagent, initially present in its dried form, is only gradually dissolved into the sample (Fig. 3b, ii). Finally, the dissolved reagent starts migrating through the device together with the flowing sample liquid (Fig. 3b, iii). As is clear from this mechanism, continuous sample liquid transport is required to achieve the current SRD system. In the absence of the thin wax barrier, no liquid droplet is formed on the sample pad, due to the occurrence of bulk sample flow caused by the large flow path width. This fast bulk liquid flow leads to saturation of the entire device with liquid within 100 s (Fig. 2b, “100 s” panel), and thus the driving force for liquid transport is eliminated before sufficient dissolution of the pre-deposited reagent. Once the entire device is saturated by the sample solution, the rehydrated model reagent (Evans Blue...
The expected reaction scheme of the LF-based one-step ELISA is shown in Fig. 4b. After sample application, the analyte is first transported by capillary forces (Fig. 4b, i) and forms the immune-complexes with the alkaline phosphatase-labeled antibodies at the conjugate pad (Fig. 4b, ii). While these complexes and the remaining non-analyte-conjugated secondary antibodies are transported to the test and control lines, re-hydration of the pre-deposited enzyme substrate into the sample liquid proceeds at the sample pad (Fig. 4b, iii). Thanks to the SRD system, the BCIP/NBT substrate is lastly transported to the test and control lines, resulting in blue-purple color development (Fig. 4b, iv). In this chromogenic reaction, BCIP is first hydrolyzed in the presence of alkaline phosphatase (ALP), and the product reduces NBT to the colored precipitate providing the colorimetric signal.

As described in the previous section, the absence of the thin wax barrier leads to insufficient transportation of the pre-deposited reagent on the sample pad, due to bulk flow of the sample liquid through the device. Therefore, no colorimetric signal generation is expected on LFT devices without the thin wax barrier on the sample pad.

**Effect of the amount of ALP-labeled antibodies**

First, the influence of the deposited amount of ALP-labeled secondary antibodies was evaluated. For this purpose, three different dilutions of the as-received anti-mouse IgG-ALP conjugate stock solution (1000-fold, 5000-fold and 30000-fold dilution) were tested. The results obtained with each dilution are shown in Figs. 5a, 5b and 5c, respectively. The largest evaluated amount of the ALP-labeled secondary antibody (i.e. 1000-fold dilution) resulted in a high background signal, as indicated by the purple color observable on the entire nitrocellulose strip (Fig. 5a). On the other hand, 30000-fold dilution led to no color development on the test line at 1 μg mL⁻¹ of IgG sample concentration, due to insufficient supply of the ALP enzyme. Therefore, the 5000-fold dilution was selected as the optimal concentration of the ALP-labeled secondary antibody in subsequent experiments.

The purple color observed on the conjugate pads after assay completion is attributed to the chromogenic enzymatic reaction caused by residual anti-mouse IgG-ALP conjugates. Despite the blocking treatment of the conjugate pad, incomplete release of the ALP-labeled secondary antibodies was inevitable.
However, it is important to note that with the developed wax-assisted SRD system, an ELISA procedure requiring just one single sample pipetting step has been successfully achieved on an LFT device.

**Effect of the enzyme substrate amount**

The influence of the amount of the BCIP/NBT enzyme substrate deposited on the sample pad has been investigated. For this purpose, the number of pipetting cycles of the substrate solution (2 μL) has been varied between 1 and 3. The corresponding results are shown in Figs. 6a, 6b and 6c, respectively. The quantitative evaluation of the optical signal obtained from the test line is shown in Fig. 6d. Pipetting of the substrate solution onto the sample pad in small aliquots of 2 μL is important to avoid the undesired spreading of the applied reagent.

The deposition of totally 4 μL and 6 μL of enzyme substrate resulted in higher achievable maximum optical signal as compared to that of 2 μL. Since more homogenous test lines (Fig. 6b) and higher sensitivity (Fig. 6d) have been obtained in the case of depositing a total of 4 μL, the pipetting of 2 μL substrate solution two times was adopted as the optimum throughout this work.

**One-step mouse IgG immunoassay with wax-modified sample pad**

The importance of the thin wax barrier on the performance of the sample pad has been confirmed first. As in the case of the dye solution experiments described above, the absence of a thin wax barrier prevented sequential assay component delivery, as confirmed by the missing control and test lines (Fig. S3, Supporting Information). This result is attributed to the failure of chromogenic enzyme substrate transport to the nitrocellulose membrane, as also observed for the dye flow in Fig. 2b, and therefore, clearly indicates the importance of the wax-assisted SRD system for the ELISA LFT.

A blocking treatment of the sample pad with 10 μL of 5%(w/v) BSA in Tris (pH 8.0, 50 mM) containing 1 mM MgCl₂ allowed to lower the detection limit for the mouse IgG model analyte by mitigating the adsorption of the analyte antigen to the sample pad. As shown in Fig. 7a, development of a test line has been visually confirmed down to a concentration of 20 ng mL⁻¹ of mouse IgG, in contrast to the much higher minimum detectable IgG concentration in the absence of sample pad blocking (500 ng mL⁻¹ in Figs. 6b and 6c).

The calibration curve for mouse IgG obtained from the LFT devices in Fig. 7a is shown in Fig. 7b. The calculated LOD (3σ method) of 15.8 ng mL⁻¹ is comparable to that achieved by a commercial microtiter plate-based ELISA method (10 ng mL⁻¹). The current wax-assisted LFT device is a promising approach to ELISAs in LF format, since in analogy to conventional LFTs, it requires only a single sample solution application with no additional user intervention.

**Conclusions**

A new type of sequential reagent delivery system requiring only a single sample application applicable to conventional lateral-
flow test devices has been developed, where the original sample pad has been replaced by a thin wax barrier-modified filter paper substrate. The essential role of this thin wax barrier modification has been confirmed based on a model experiment using dyes as well as a mouse IgG ELISA. Under optimized conditions, the elaborated device achieved a limit of detection of 15.8 ng mL⁻¹, which is comparable to the conventional ELISA method in a microplate, but avoiding the complicated assay procedures and long assay times associated with the latter one. It is assumed that the developed LFT device with integrated SRD is applicable to a wide class of ELISAs by using different antibodies or by altering the combination of labeling enzymes and substrates.

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Supporting Information

Supporting information includes schematics of the preparation of test and control lines using a desktop electronic plotter/cutter; detailed structure of the LFT device for the wax-assisted SRD targeting one-step ELISA; and result of the mouse IgG ELISA on LFT devices without the thin wax barrier. This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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