Biosensors and Bioelectronics 102 (2018) 27–32

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

# Development of an automated wax-printed paper-based lateral flow device for alpha-fetoprotein enzyme-linked immunosorbent assay

Pattarachaya Preechakasedkit[a], Weena Siangproh[b], Nanthika Khongchareonporn[c], Nattaya Ngamrojanavanich[c,d,*], Orawon Chailapakul[d,e,*]

[a] Program in Biotechnology, Faculty of Science, Chulalongkorn University, Patumwan, Bangkok 10330, Thailand
[b] Department of Chemistry, Faculty of Science, Srinakharinwirot University, Wattana, Bangkok 10110, Thailand
[c] The Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Patumwan, Bangkok 10330, Thailand
[d] Electrochemistry and Optical Spectroscopy Center of Excellence (EOSCE), Department of Chemistry, Faculty of Science, Chulalongkorn University, Patumwan, Bangkok 10330, Thailand
[e] Center for Petroleum, Petrochemicals and Advanced Materials, Chulalongkorn University, Patumwan, Bangkok 10330, Thailand

ARTICLE INFO

*Keywords:*
Paper-based lateral flow device
Enzyme-linked immunosorbent assay
Wax-printing method
Alpha-fetoprotein
Point-of-care testing

ABSTRACT

In this study, a novel wax-printed paper-based lateral flow device has been developed as an alternative approach for an automated and one-step enzyme-linked immunosorbent assay (ELISA). The design pattern consisted of a non-delayed channel, a wax-delayed channel, a test zone and a control zone. This system was easily fabricated on a nitrocellulose membrane using a wax-printing method and then baked in an oven at 100 °C for 1 min. The four barriers of the wax-delayed channel could delay the flow time for 11 s compared to the flow time of the non-delayed channel. To use the device under optimal conditions, alpha-fetoprotein (AFP) was detected at a limit of detection of 1 ng $mL^{-1}$ and assessed with the naked eye within 10 min. A colorimetric intensity was also measured using a smart phone and computer software at a linear range of 0.1–100 ng $mL^{-1}$ with a good correlation. Furthermore, the proposed device was successfully applied to detect AFP in human serum. Therefore, the wax-printing demonstrates a user-friendly, easy and quick method for the fabrication of the device, which could be used as a one-step, portable, disposable, low-cost, simple, instrument-free and point-of-care device for the automated ELISA.

## 1. Introduction

The enzyme-linked immunosorbent assay (ELISA) has been widely used as an analytical technique in many applications, especially in clinical diagnosis, because of its key advantages, namely high specificity and sensitivity (Felin et al., 2017; Liu et al., 2017a, 2018; Yu et al., 2017). Initially, the conventional ELISA is performed in a 96-microwell plate and requires complicated steps, long analysis time and high volume of reagents (Damle et al., 2017; Lei et al., 2017; Li et al., 2017; Liu et al., 2017b). To reduce analysis time and volume of reagents, in 2010, Cheng and co-workers firstly reported a paper-based ELISA by fabricating hydrophobic and hydrophilic areas on a filter paper using photolithography (Cheng et al., 2010). The layout of paper-based ELISA was similar to the plastic 96-microwell plates. The use of the paper-based ELISA could successfully reduce analysis time (51 min) and volume of reagents (12 μL) compared to the conventional ELISA (213 min of analysis time and 300 μL of reagents volume). However, this paper-based ELISA still required complicated steps (at least 4 steps).

To overcome this drawback, a lateral flow immunoassay (LFIA) has been proposed as a one-step and rapid assay to simplify the conventional ELISA and shorten the analysis time (Quesada-Gonzalez and Merkoci, 2015; Sajid et al., 2015). Normally, gold nanoparicles have been traditionally used as a label for the LFIA, and the traditional gold nanoparticles-based LFIA still provided low sensitivity compared to enzyme label (Bahadır and Sezgintürk, 2016; Samsonova et al., 2015). In 2012, Parolo and co-workers improved the sensitivity of the gold nanoparticles-based LFIA by increasing the sizes of sample and conjugate pads. Although 3-fold increase in the width of the pads demonstrates 8-fold sensitivity improvement, a large amount of sample volume (600 μL) is required. Furthermore, there is one of alternative methods for improving sensitivity of the traditional LFIA by a LFIA combining with ELISA (LFIA-ELISA) (Gao et al., 2014; Shu et al., 2017; Zou et al., 2012). Unfortunately, colorimetric signals resulting from ELISA cannot be directly detected when using only an enzyme-labeled antibody (Hsu et al., 2014; Shih et al., 2015). An additional step of the addition of a substrate solution is also required to produce the

* Corresponding authors.
*E-mail addresses:* nnattaya@chula.ac.th (N. Ngamrojanavanich), corawon@chula.ac.th (O. Chailapakul).

http://dx.doi.org/10.1016/j.bios.2017.10.051
Received 8 September 2017; Received in revised form 30 October 2017; Accepted 31 October 2017
Available online 01 November 2017
0956-5663/ 

colorimetric signal (Khan et al., 2015; Wang et al., 2012; Wu et al., 2017). Consequently, the conventional LFIA-ELISA requires at least 2 steps by loading a sample solution on the device until reaction completion and then immersing the device in the substrate solution for colorimetric evaluation (Kim and Pyun, 2009; Wang et al., 2017). Additionally, the conventional LFIA-ELISA needs the washing steps before immersing the device in the substrate solution to remove the residue enzyme, which may cause the high background color on the device (Ozalp et al., 2013). Therefore, the development of an automated LFIA-ELISA is a potential and interesting approach for overcoming the above drawbacks.

The automated sandwich LFIA-ELISA has been firstly developed by direct fabrication of a delayed channel on a nitrocellulose membrane (NCM) using inkjet printing (Apilux et al., 2013). The organic solvent, including dipropylene glycol methyl ether acetate containing acrylic polymer, was used as solvent ink for patterning the delayed channel on the NCM. Although the automated device was successfully developed, this method still required solvent ink, which is a hazardous chemical for human health and an environmental contaminant. Another delayed-release sandwich LFIA-ELISA using a substrate pad attached to an asymmetric polysulfone membrane has also been reported for the automated assay (Joung et al., 2014). The substrate pad was prepared with complicated steps before being attached to the asymmetric polysulfone membrane. Then, the substrate pad attached to the asymmetric polysulfone membrane was stacked on the NCM. However, the limitations of this delayed-release technique are the use of complicated steps and the need for several types of materials to fabricate the delayed-release device.

Interestingly, a wax-printing method has been widely used to fabricate the paper-based devices because of several benefits, including low cost, ease of fabrication and no need of organic solvents (Carrilho et al., 2009; Ge et al., 2012; Renault et al., 2014). In 2010, Lu and co-workers firstly reported the fabrication of various patterns on the NCM using wax-printing method (Lu et al., 2010). The device consisting of 3 mm diameter as detection zones was applied for a dot immunoassay. The dot immunoassay on this device still needed complicated steps (4 steps). The first time for the use of wax-printed pillars as delay barriers on the LFIA was reported by Riva and co-workers (Rivas et al., 2014). The existent wax-printed pillars could increase binding reaction times between an analyte and a gold nanoparticles-labeled antibody leading to the sensitivity improvement. Another improvement of LFIA has also been reported by fabricating three-dimensional paper-based devices using wax printing (Han et al., 2016). The devices were designed with multiple layers (5 layers) which each layer contained different reagents for immunoassay procedures and signal enhancement. This designed device can improve sensitivity of gold nanoparticles label. Although the wax-delayed barriers were used for improving sensitivity of the conventional LFIA, there is no report about the use of the wax-delayed barriers for developing the automated LFIA-ELISA.

In this study, a wax-printed paper-based lateral flow device has been developed as a novel, alternative, automated, one-step and instrument-free LFIA-ELISA. Our proposed device provides the advantages above the conventional LFIA-ELISA and paper-based ELISA in term of short analysis time and single-loading sample. In addition, the proposed device is able to provide higher sensitivity over the traditional gold nanoparticles-based LFIA. The required design of the delayed barrier was directly fabricated on the NCM using the wax-printing method. The assay parameters were optimized, including the concentration of the immobilized antibody, the ratio of the enzyme-labeled antibody and the ratio of the substrate to obtain an optimal device. To demonstrate the usefulness of the proposed device, alpha-fetoprotein (AFP) was selected as an appropriate analyte. AFP is well-known as a key tumor marker for the early diagnosis of patients with liver cancer (Wang et al., 2014a, 2014b). Normally, the maximum level of AFP in healthy human is lower than 25 ng $mL^{-1}$, and the abnormally increased levels of AFP can cause various cancerous diseases such as hepatocellular cancer, yolk sac cancer, liver metastasis from gastric cancer, testicular cancer, and nasopharyngeal cancer (Liu et al., 2015; Siegel et al., 2016). Therefore, the proposed device for rapid screening of AFP levels acts as a great significance for early cancer diagnosis and treatment. Furthermore, the data analysis using this device could be easily obtained from qualitative measurement by the naked eye and quantitative measurement through a smart phone and computer software.

## 2. Materials and methods

### 2.1. Chemicals and materials

Bovine serum albumin (BSA), glycine, sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium choloride, Tris(hydroxymethyl)aminomethane, casein, magnesium chloride and Tween 20 were purchased from Sigma Aldrich (St. Louis, MO, USA). Alpha-fetoprotein (AFP), AFP monoclonal antibodies (10–1390 as a secondary antibody and 10–1391 as a primary antibody) were purchased from Fitzgerald (North Acton, MA, USA). Alkaline phosphatase (ALP) conjugation kit was purchased from Abcam (Cambridge, UK), BCIP/NBT (5-bromo-4-chloro-3′-indolyphosphate *p*-toluidine salt, nitro-blue tetrazolium chloride) substrate and substrate buffer solutions were purchased from Nacalai Tesque (Tokyo, Japan). Goat-antimouse IgG (GAM) was purchased from Jackson ImmunoResearch (West Grove, PA, USA). Standard pooled human serum was purchased from Kohjin Bio (Saitama, Japan). All solutions were prepared using 18 Ω milli-Q water. Nitrocellulose membrane (AE100), glass fiber membrane (Standard 17) and absorbent pad (CF7) were purchased from Whatman-GE healthcares (Pittburgh, PA, USA).

### 2.2. Reagents preparation

Firstly, GAM was prepared to the concentration of 0.5 mg $mL^{-1}$ using 0.01 M PBS buffer (pH 7.4), and the concentration of the AFP primary antibody (AFP 1° Ab) was varied using the same buffer. The AFP secondary antibody (AFP 2° Ab) was conjugated to ALP using ALP conjugation kits to be used as the enzyme-labeled antibody (AFP 2° Ab-ALP), and the optimal amount of the enzyme-labeled antibody were optimized. The BCIP/NBT substrate was diluted using the substrate buffer at various ratios to select an optimal value. The blocking buffer was 100 mM Tris buffer (pH 9.2) with 1% (w/v) casein, and the washing buffer was 0.01 M PBS (pH 7.4) containing 0.05% Tween 20. 100 mM Tris buffer (pH 9.2) was used as running buffer and diluted buffer of AFP.

### 2.3. Design of the wax-printed paper-based lateral flow device for sandwich ELISA

The design of the device for the sandwich ELISA is shown in Fig. 1A. The components of the device consisted of three pads including sample, wax-printed and absorbent pads. The key concept of the wax-printed pad was the control of the reagent flow to complete the sandwich ELISA using single-loading sample. The wax-printed pad comprised of a non-delayed channel and a wax-delayed channel before merging into one main channel of a detection region containing test and control zones. The wax-delayed channel was specially designed with four barriers to delay the solution flow before passing a substrate region while the solution directly flowed through the non-delayed channel into an enzyme-labeled antibody region. At the detection region, the positions of the test and control zones were respectively labeled with T and C alphabets.

### 2.4. Fabrication of the wax-printed paper-based lateral flow device

The pattern of the wax-printed pad was designed using the Adobe Illustrator program. The designed pattern was printed on the NCM using a wax printer (Xerox ColorQube 8570, Japan). After printing, the