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Research Article

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Assay time reduction and thermal stability improvement of a low-cost, wax-dipping paper-based microfluidic device

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ABSTRACT

Lab-on-paper or paper-based microfluidic devices (µPADs) are an alternative technology for clinical diagnosis and point-of-care testing, particular in developing countries. In this report, a simple and facile wax dipping method for the fabrication of paper-based microfluidic devices was described,and its feasibility in terms of decreased assay time and improved thermal stability was demonstrated. Multiple wax-based products, including beeswax (BW), microwax (MW), paraffin (PF) and polyethylene wax (PE), can be used in the wax dipping method to improve the thermal stability of the devices. Beeswax and PE were selected as suitable ingredients in terms of their ability to improve the thermal stability. The optimal melting temperature of beeswax containing PE was in the range of 140-160 °C with an optimal dipping time of 5 seconds. The results showed that the use of PE as an additive could help improve the thermal stability of the µPAD,which could withstand temperatures of up to 45 ºC for 15 minutes without affecting the channel resolution. In addition, wax dipping was used to improve the fluid flow of a µPAD made from a paper towel and reduced the assay time 5-fold.

Keywords: Lab-on-paper, paper-based microfluidic devices (µPADs), wax-based products, wax dipping, thermal stability, papertowel

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INTRODUCTION

The prevention and treatment of diseases is crucial in both developed and developing countries. Thus, diagnostic technologies are important tools for improving the health care of people around the world.However, most diagnostic devices are expensive, large and require trained personnel; these features make diagnostic devices unsuitable for use in developing countries [1-3]. Therefore, diagnostic devices that are simple, inexpensive and portable are needed. Currently, paper-based microfluidic or lab-on-paper devices, also called microfluidic paper-based analytical devices (µPADs), are novel tools that are constructed by patterning paper into point-of-care diagnostic devices. Due to several advantages, such as speed, ease of use, low cost, small sample and reagent volumes, portability, disposability and the possibility of performing multiple analyses on a single device [1-4, 5, 6], µPADs can serve as analytical devices that are an alternative technology for multiplex clinical diagnosis and point-of-care testing, especially for resource-limited countries. Furthermore,unlike the flow systems or FIA systems [7, 8], µPADs can be operated without external equipment or pumping to move fluid because the fluid flow in µPADs is controlled by capillary forces [1, 3, 6].

Recently, several methods of µPAD fabrication have been developed, including photolithography [1, 9], plotting [10], inkjet etching [5, 11], plasma etching [12], cutting [13], wax printing [14, 15] and wax screen-printing [16]. Although these techniques can be used to pattern µPADs, each fabrication method has some drawbacks. For example, the hydrophilic channels in µPADs fabricated using photolithography, inkjet etching or plasma etching methods are exposed to polymers and solvents, which may interfere with colorimetric or electrochemical detection on µPADs [16]. However, when using plotting, cutting, wax printing and wax screen-printing techniques, the hydrophilic areas are not exposed to chemicals or solvents. The plotting method requires a customized plotter [3, 11], and the cutting technique requires tape to bond the layers of the device [3, 14]. Moreover, wax printing and wax screen-printing result in poor resolution of the hydrophilic channels [14, 16]. Therefore, a simple fabrication method that does not require chemicals or solvents and provides good resolution of the hydrophilic channels is required.

Wax dipping is a simple method for the fabrication of paper-based microfluidic devices because the procedure requires only a single dipping step to pattern the paper, as described in a previous report [17]. Wax is an inexpensive material that is generally used worldwide. The wax dipping method requires only a hot plate to control the temperature of the melted wax. Using the wax dipping method, devices can be patterned in 1 minute with good resolution of the hydrophilic channels [17]. Moreover, the hydrophilic area is not exposed to chemical compounds or solvents.

Because of the nature of the wax dipping procedure, the method allows multiple sheets of paper to be attached without the use of other supplemental equipment (e.g., combination with a blood separation membrane for plasma separation) [18]. Additionally, multiple wax products can be mixed together based on the desired properties or characteristics of the device.

Rapid analysis is very necessary for some clinical tests, especially in emergency cases [19]. Although performing an analysis on paper is accepted as a rapid test and the results can be collected within minutes, problems are still encountered when using this method to analyze viscous samples (e.g., high hematocrit blood samples). The blood cannot penetrate through the porous paper, which leads to problems with clotting on the paper. The use of only a regular Whatman No.1 filter paper may slow the reaction, and worse blood clotting can occur. To speed up the assay, a sheet of fast-absorbing material, such as a paper towel, may be added to the Whatman No.1 paper. Flow control in µPADs can decrease the detection time of the multistep analysis on the paper without adding to the expense of the devices [20]. However, wax-based devices have a limitation in terms of thermal stability. High temperatures, common in many developing countries, can affect the wax barrier of wax-based µPADs. Thus, methods to improve the thermal stability of low-cost µPADs must be explored. Because the wax dipping method can be performed with a mixture of multiple types of wax-based products, the desired temperature stability of the µPAD can be obtained based on the wax composition. In this paper, we demonstrated the feasibility of wax dipping for increasing the thermal stability of µPADs and shortening the assay times.

EXPERIMENTAL SECTION

Materials and Chemicals

Whatman No.1 filter paper was purchased from Whatman International Ltd. (Maidstone, England). Iron molds were made by a laser cutting shop. White Beeswax (BW), microwax (MW), micro paraffin wax (MP), polyethylene wax (PE), food coloring, paper towels and magnetic bars were purchased from a local shop (Bangkok, Thailand). Glass slides were supplied by Sail Brand (Jiangsu, China). The hot plate (C-MAG HS 7) was a product of IKA®(Wilmington, USA). The scanner (HP Deskjet F300) was from HP.

Lab-on-paper devices fabricated by wax dipping

A wax dipping method was utilized to fabricate lab-on-paper devices in a previous study[17]. The procedure for wax dipping is shown in Fig. 1. Briefly, an iron mold for wax dipping was created using a laser cutting technique. To fabricate an assembly mold, a piece of Whatman No.1 paper was placed on a glass slide, and a permanent magnet was placed on the back of the glass slide. Then, the iron mold was overlaid on the paper and was temporarily attached due to the magnetic force from the permanent magnet on the opposite side of the glass slide (Fig. 1A). For the wax dipping procedure, white beeswax pellets were melted on a hot plate, and the temperature was maintained in the range of 140-160 °C using an electronic contact thermometer (IKA$^{®}$ETS-D5). Then, the assembly was dipped into a chamber of melted wax for 5 seconds and lifted off (Fig. 1B). After the wax cooled to room temperature, the paper was removed from the glass slide and the iron mold was separated from the paper (Fig. 1C). Finally, the lab-on-paper device was ready for use.

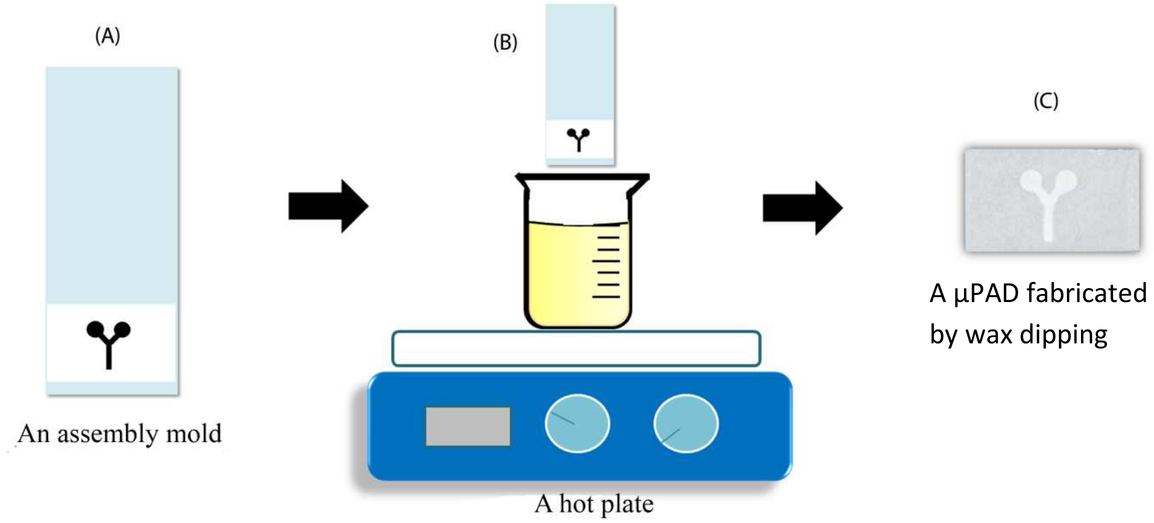


Fig. 1 The µPAD fabrication processes using the wax dipping method. (A) An assembly mold was produced using a magnet, a glass slide, paper and an iron mold; (B) the wax dipping setup; (C) the completed µPAD separated from the assembly mold

Optimal temperature of wax-based products in the wax dipping method

Wax-based products have different melting points and optimal dipping temperatures. In this study, beeswax, microparaffin wax and microwax were tested to create µPADs at different temperatures in the range of 100-160 °C. Then, 10% polyethylene (PE) wax was added to the beeswax, microparaffin wax ormicrowax and used for µPAD fabrication. The optimal dipping temperatures of the beeswax-PE, microparaffin wax-PE, and microwax-PE were studied in the range of 100-160 °C. The optimal temperature for µPAD fabrication with each type of wax was the range of temperatures that can create high resolution between the hydrophobic and hydrophilic areas on the paper. The optimal temperature for each wax-based product was used to create a µPAD,and the thermal stability of each µPAD was determined.

The effect of the polyethylene wax concentration on the thermal stability of the µPAD

Polyethylene wax was used to increase the melting point of the wax-based products and increase the thermal stability of the µPAD. Thus, the effect of the concentration of PE in the beeswax was evaluated. Polyethylene wax was added to beeswaxat various concentrations rangingfrom 0-50% to construct the µPAD. The iron mold was placed on Whatman No.1 paper and dipped in melted wax that contained 0, 5, 10, 20, 30, 40 and 50% PE at the optimal temperature for 5 seconds. Finally, the thermal stability of the µPADswasdetermined.

Thermal stability test

After the fabrication step, the thermal stability of eachµPAD was tested. First, the µPAD was scanned using a scanner, and the widths of the hydrophilic channels on the µPAD were measured using the ImageJ program (National Institutes of Health). Then, the thermal stability of the µPAD was determined by placing the µPAD on a hot plate or in an oven at different temperatures (range, 30-65 °C)for different times (range, 1-15 minutes). Next, food coloring was dispensed onto the hydrophilic area of the µPAD. An image of the µPAD was captured using a digital camera after the food coloring dried. The widths of the hydrophilic channels on the device after the heat treatment were measured using ImageJ. The width of the hydrophilic channels before and after the thermal stability tests were compared and analyzed using a paired samples *t*-test.

RESULTS AND DISCUSSION

1. Optimal temperature of the wax-based products for wax dipping

In addition to beeswax, other wax-based products that are commonly used in aroma candles, including microwax, microparaffin wax and polyethylene wax, were investigated to determine their ability to improve the thermal stability of µPADs. Due to the properties of wax-based products, different types of wax have different melting temperatures. Moreover, the temperature of the wax affects the resolution of the hydrophilic channel on the paper. Therefore, the optimal melting temperature of the wax-based products for µPAD fabricationwas tested. As shown in Fig. 2, different waxes and wax combinations, including micro paraffin wax, micro paraffin-PE wax, microwax, microwax-PE, beeswax, and beeswax-PE, were used to fabricate µPADs at different temperatures in the range of 100-160 °C.

The results show that microparaffin wax can be used for fabrication at the lowest temperatures (110-135 °C). For fabrication using microwax, the optimal temperature for fabrication covers a wide range (120-160 °C). For beeswax, the optimal dipping temperature is in the range of 120-130 °C. When the temperature is below the optimal range, the hydrophobic and hydrophilic areas are not completely separated. In contrast, using a dipping temperature above the optimal range causes excessive spreading of the wax into the paper. When PE is used as an additive, the melting temperature is higher than that of the wax-based products without PE. Based on the results shown in Fig. 2, the optimal temperatures for micro paraffin-PE, microwax-PE and beeswax-PE are in the ranges of 125-160, 130-160 and 140-160 °C, respectively. Based on these results, beeswax-PE was selected for the fabrication of the µPAD because it has the highest dipping temperature. This condition may affect the thermal stability of the µPAD. Compared to the wax dipping method described in a previous report [17], the optimal dipping temperature of wax formulationscontaining PE as an additive is higher than that of wax formulations that contain only one type of wax. Here, the optimal dipping temperature increased from 120-130 °Cto 140-160 °Cwhen PE was mixed with the wax. It can be concluded that PE could help improve the thermal resistance of wax-based products. For this reason, we expect that the µPADs created using wax with PE as an additive will have increased thermal stability.

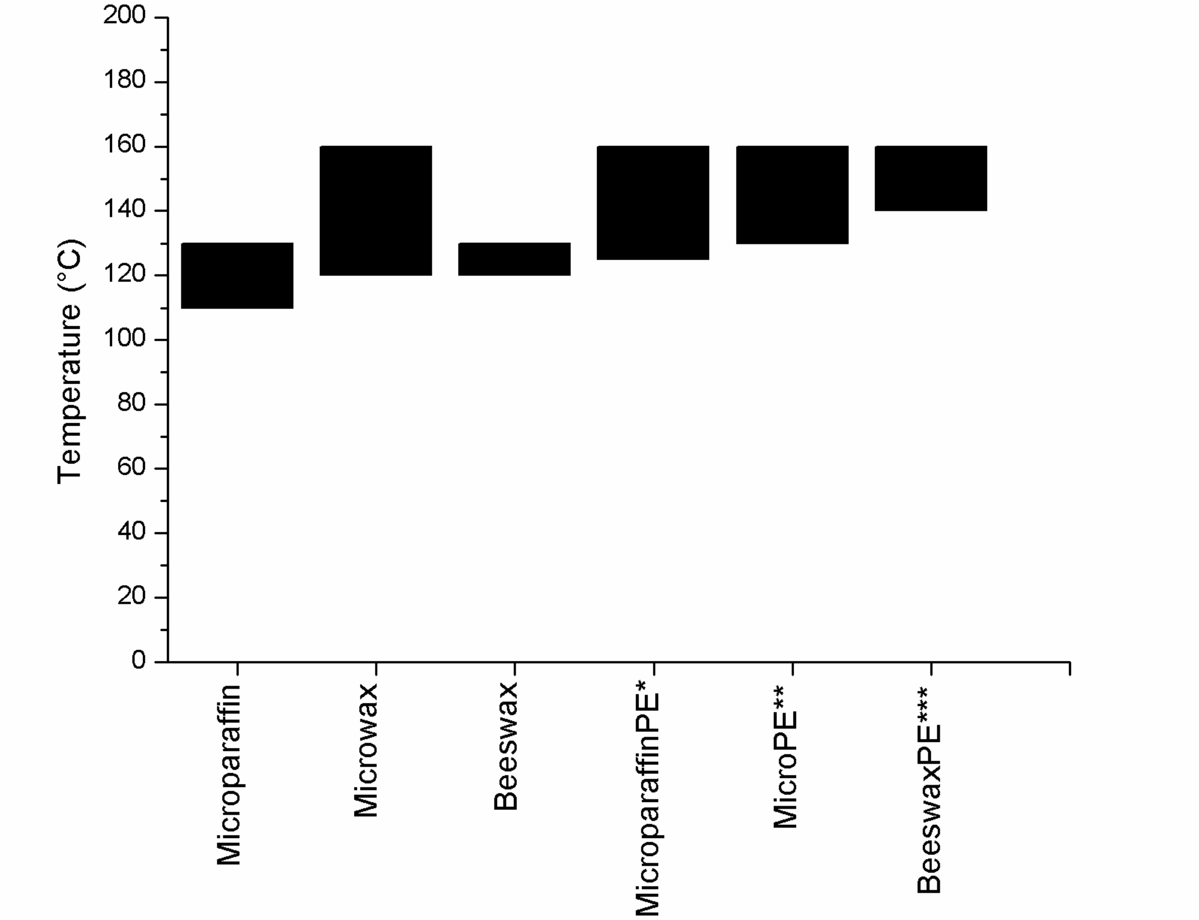


Fig. 2 The optimal dipping temperature of the wax-based products

** paraffin:microwax 10:3 and polyethylene wax (10% of the paraffin weight)*

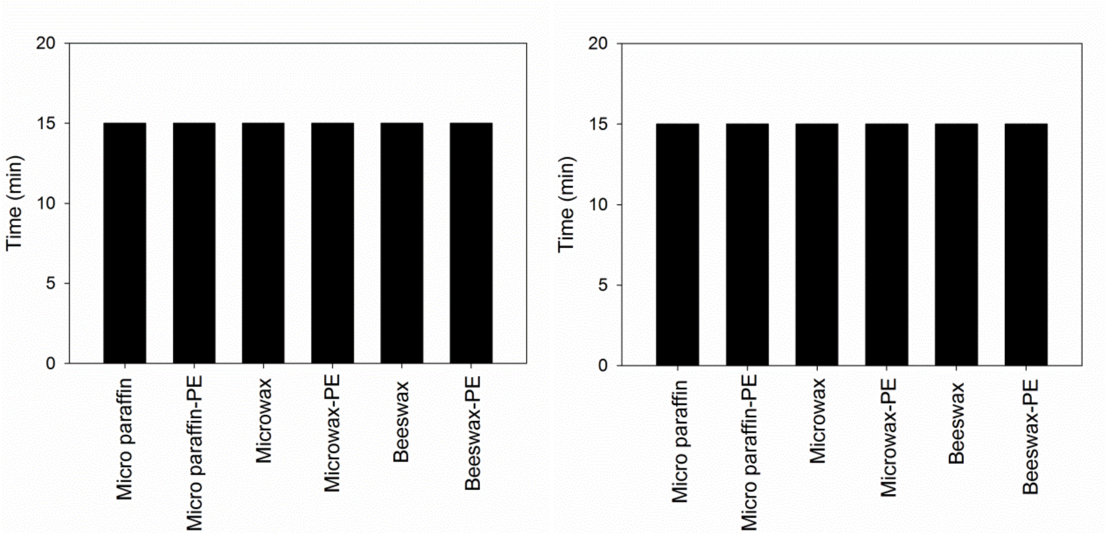
***microwax and polyethylene wax (10% of themicrowax weight)*

**** beeswax and polyethylene wax (10% of the beeswax weight)*

2. Thermal stability of the µPAD

It is generally known that wax is not stable when exposed to high temperatures. Therefore, µPADs based on wax ingredients have some limitations when used outside the laboratory, especially during in-field assays in countries located in tropical regions. To improve the thermal stability of wax-based µPADs, wax-based products used to produce aroma candles were tested. After fabricating µPADs using different wax-based products, each µPAD was placed on a hot plate at 30, 35, 40 and 45 °Cfor 1, 5, 10 and 15 minutes and tested in quadruplicate at each temperature and time. Then, the average width after heating on a hot plate was compared to the width of the device before heating.

The results shown in Fig. 3A and 3B indicate that µPADs fabricated using all types of wax could withstand temperatures up to 35 °Cfor 15 minutes. The µPADs fabricated using beeswax and beeswax-PE could withstand a temperature of 40 °Cfor 15 minutes, while the µPADs fabricated using micro paraffin and micro paraffin-PE, microwax, and microwax-PE could withstand the temperature for 1, 5 and 10 minutes, respectively(Fig. 3C). At 45 °C (Fig. 3D), the device fabricated using micro paraffin could no longerwithstand the temperature. Similarly, devices constructed using micro paraffin-PE, microwax, microwax-PE and beeswax could withstandthis temperature for only 1 minute. In contrast, using PE as an additive to beeswax helped improve the thermal stability of the µPAD such that it could withstand temperatures of up to 45 °Cfor 10 minutes. It can be concluded that PE can help improve the thermal stability of the µPAD, and beeswax-PE is suitable for the wax dipping method in terms of thermal stability.


(A) Temperature of 30 °C (B) Temperature of 35 °C

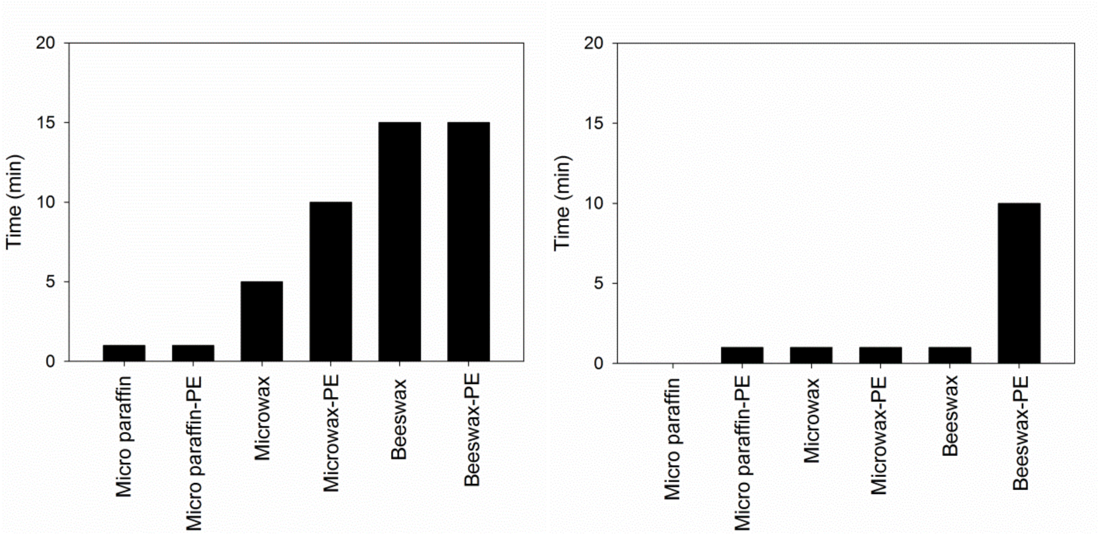

(C) Temperature of40 °C (D) Temperature of45 °C

Fig. 3 The thermal stability of µPADs fabricated using wax-based products at 30 °C (A), 35°C (B), 40 °C (C) and 45 °C(D)

3. Comparison of the thermal stabilities of µPADs constructed using beeswax and beeswax-PE

Based on the previous experiment, beeswax and beeswax-PE were found to be suitable for the fabrication of µPADs because of their high thermal stabilities. Therefore, to compare the thermal stability of the µPADs constructed using beeswax and beeswax-PE, the µPADs fabricated using both wax-based products were incubated at 30, 35, 40, 45, 50, 55, 60 and 65 °C in an oven for 15 minutes. The widths of the hydrophilic channels before and after the heat treatment were measured and compared using the paired *t*-test. The results indicate that the width of hydrophilic channel on the µPAD constructed using beeswax after heating was significantly different compared withits width before heating at 35-65 °C ($p < 0.05$). The width of the hydrophilic channel of the µPAD fabricated using beeswax-PE was significantly different at 45-65 °C ($p < 0.05$) (Fig. 4). It can be concluded that the µPAD fabricated using beeswax-PE can withstand high temperatures better than the µPAD fabricated using beeswax.

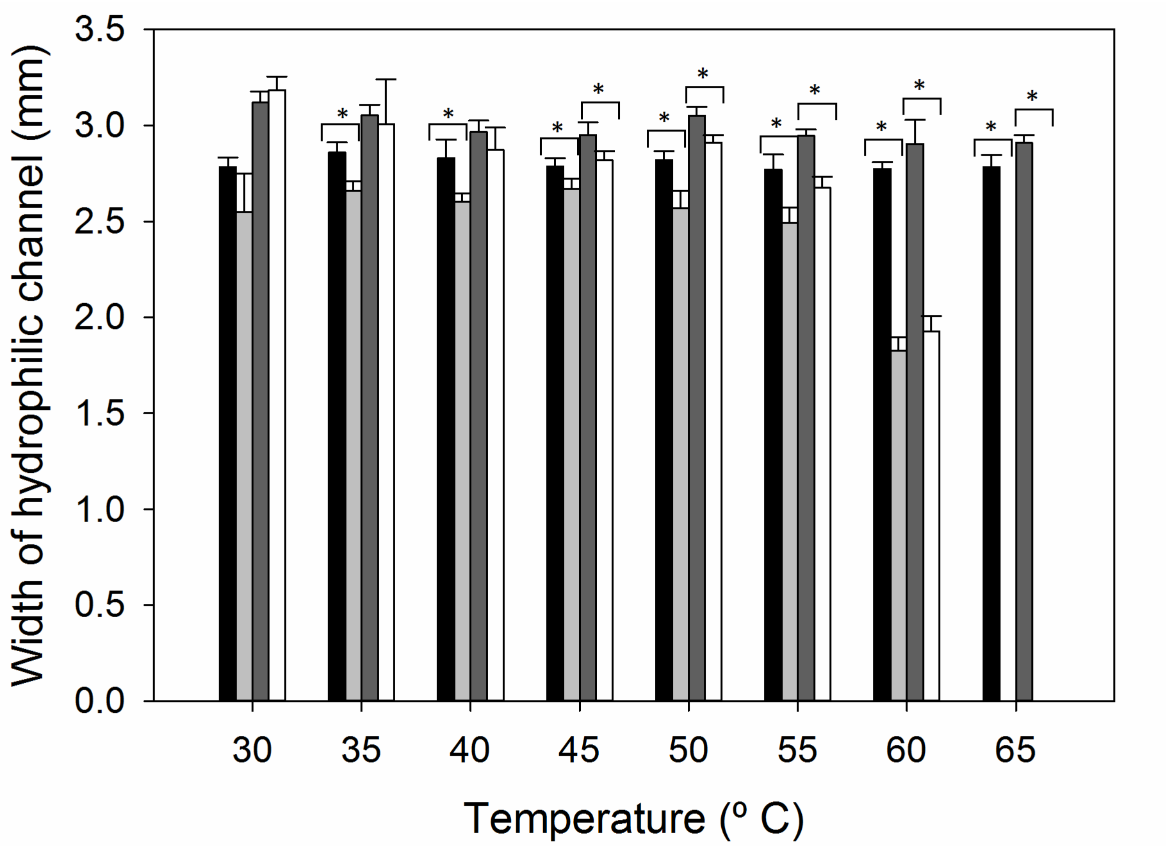


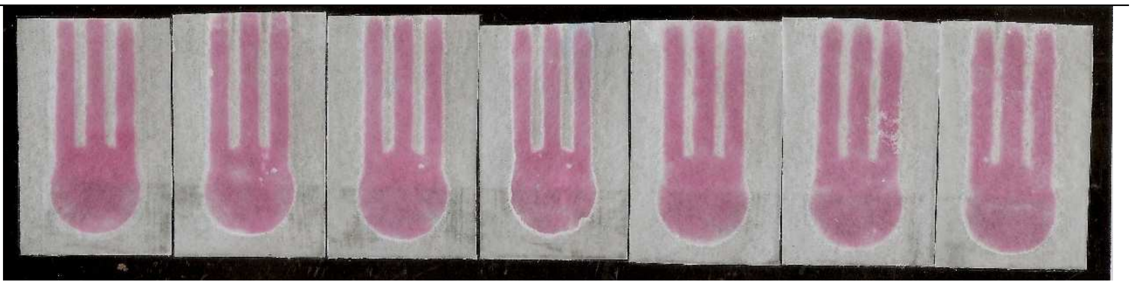
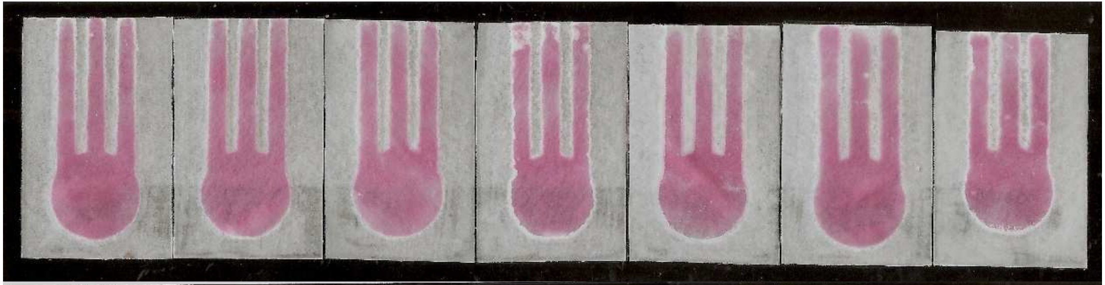
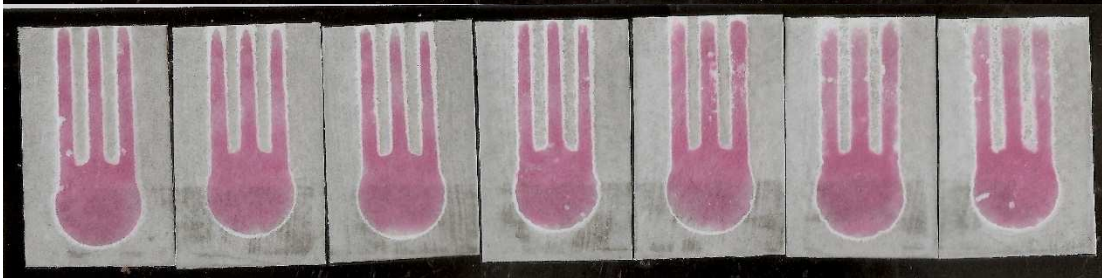
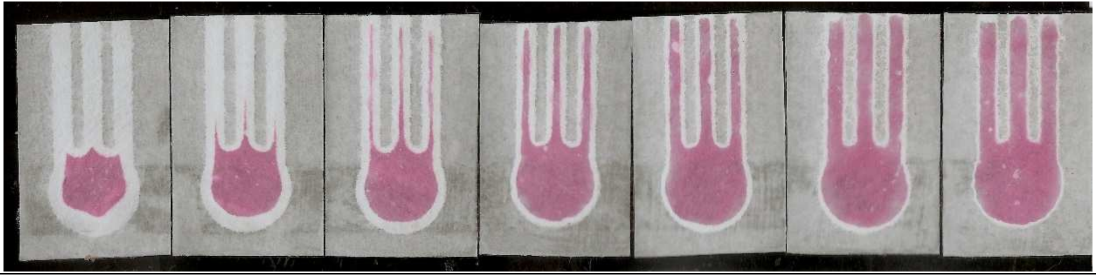
Fig.4 Width of hydrophilic channel on the µPAD constructed by beeswax and beeswaxPE before and after thermal stability test at 30-65 °C for 15 min. Paired t-test ($p < 0.05$).beeswax ■ before heat; □ beeswax after heat; ▨ beeswaxPE before heat; □beeswaxPE after heat

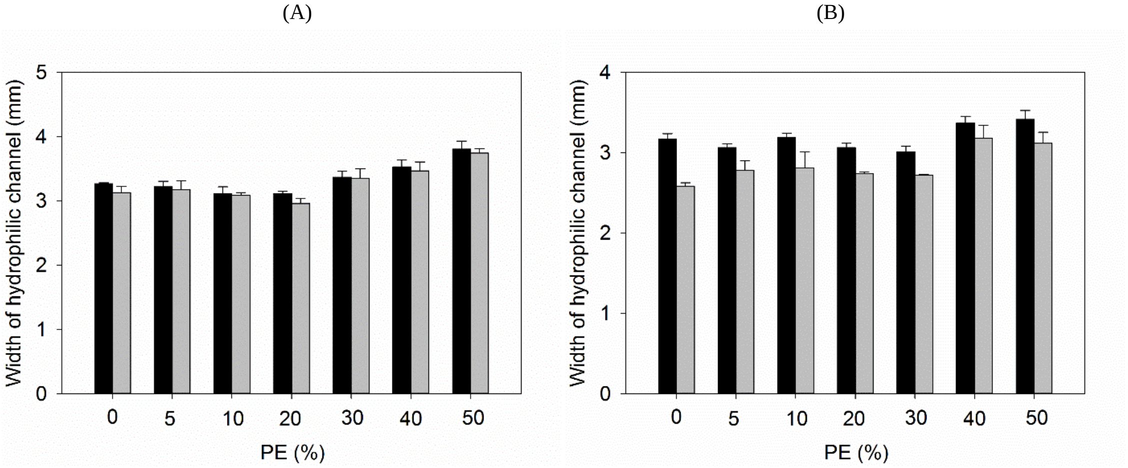
4. Effect of the PE concentration on the thermal stability of the µPAD

PE in the range of 0-50% was added to beeswax to construct the µPAD. The iron mold was placed on Whatman No.1 paper and dipped in melted wax that contained 0, 5, 10, 20, 30, 40 and 50% PE at the optimal temperature for 5 seconds. The µPADs were scanned before being placed in an oven at temperatures of 50, 55, 60 and 65 °C for 15 minutes. Then, food coloring was dispensed onto the hydrophilic channels before scanning (Table 1). The widths of the hydrophilic channels were measured using Image J.

The results in Fig. 5 show that high temperatures can melt the wax on the µPAD, resulting in narrow hydrophilic channels. Therefore, the widths of the hydrophilic channels decrease as the temperature increases. At 65 °C, it was clear that using a high PE concentration to fabricate the µPAD can increase the thermal stability of the µPAD. The µPAD fabricated using only beeswax with no added PE could not withstand the high temperatures. In contrast, increasing the PE concentration could increase the thermal stability of the µPAD. However, generating boundaries on the paper using a high PE concentration was difficult because it required high dipping temperatures (0%PE required 120-130 °C, 5-10% PE required 140-160 °C, 20-30%PE required 150-160 °C and 40-50% PE required 160-170 °C) and long dipping times (0% PE required 1 seconds and 5-50% PE required 5 seconds). The boundary of a µPAD fabricated using a high PE concentration was less sharp compared with that of a µPAD fabricated using a low PE concentration, even under optimal conditions. Based on these results, the hydrophilic channel of the µPAD created using 30-50% PE was not completely distinct from the hydrophobic area. Moreover, beeswax containing 5-20% PE could be used to create µPADs with high resolution. However, µPADs fabricated using 5% PE were not durable at high temperatures (i.e., 65 °C) because this PE concentration was too low to maintaina wax-like character at high temperatures. Therefore, 10-20% PE was optimal and suitable for increasing the thermal stability of µPADs while maintaining high resolution between the hydrophilic and hydrophobic areas.

Table 1 Thethermal stability of µPADs fabricated using different PE concentrations (0, 5, 10, 20, 30, 40 and 50%)

Temperature (°C)	Polyethylene wax (%)						
	0	5	10	20	30	40	50
50							
55							
60							
65							


(A) (B)

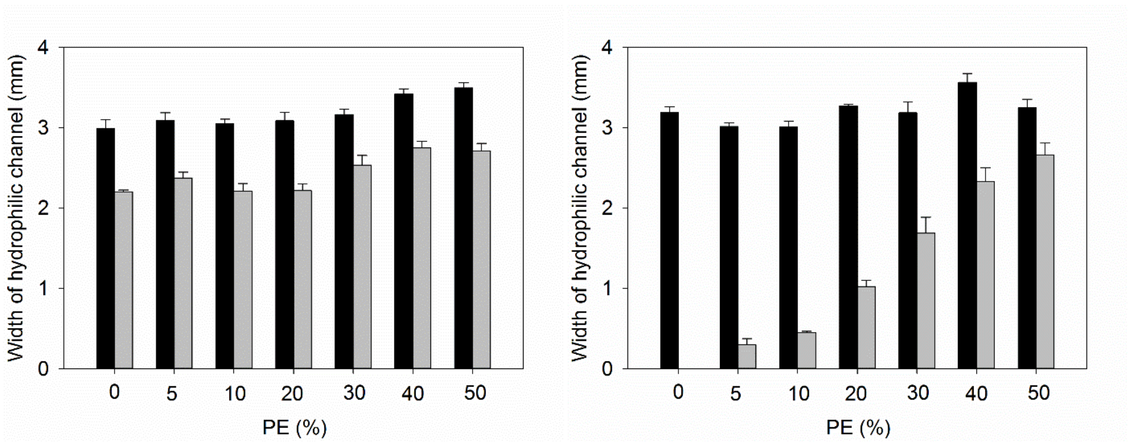

(C) (D)

Fig.5 Correlation of the width of hydrophilic channel and PE concentration after heat at (A) 50 °C (B) 55 °C (C) 60 °C and (D) 65 °C for 15 min. ■ Widthbefore heat; ▨ Width after heat

5. **Improvement in flow via the use of a paper towel on the µPAD**

It is well known that paper towels adsorbwater well. Therefore, fluids flow very rapidly on a paper towel, which can help to improve the fluid flow in a µPAD. To investigate the optimal dipping temperature for paper towels, the paper was cut into 2.50 x 2.50 cm squares and used for µPAD fabrication in a dipping temperature range of 100-160 °C. The results indicated that patterns can be created on a paper towel in the range of 110-160 °C (data not shown). Wax can be used to pattern two types of paper for µPADs. To improve the fluid flow on paper, two pieces of Whatman No.1 paper and paper towel were merged to create a µPAD (Fig. 6A) via wax dipping using beeswax containing 10% PE, and the fluid flow through the device was investigated. Food coloring was dispensed onto the first section of the device (Whatman No.1 paper), and the time until the fluid fully filled the third section of the device was recorded. To compare the fluid flow through the device, a µPAD was created that contained Whatman No.1 paper in the second sectionof the device instead of paper towel. The results showed that the fluid completely filled the third section of the µPAD fabricated using paper towel in approximately 30 seconds, while the µPAD created using Whatman No.1 paper required more than 150 seconds to completelyfill (Fig. 6B). Based on these results, paper towel could improve the fluid flow in a µPAD by 5 times when compared to Whatman No.1 paper. This setup will be useful for chemical reactions that require rapid reactions between reagents and analytes. Moreover, such rapid reactions could help improve the turnaround time of assays used for clinical diagnosis.

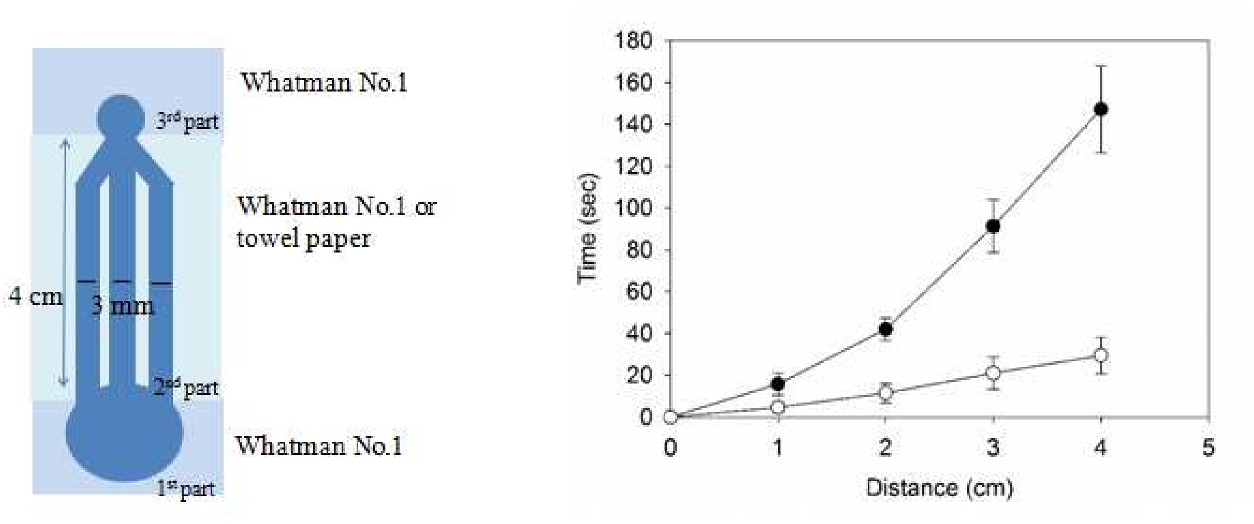

(A) (B)

Fig. 6 (A) A µPAD fabricated using three sections of paper to improve the fluid flow through the device. (B) A graph illustrating fluid flow through the µPAD.●Fluid flow usingWhatman No.1 paper; ○ fluid flow using paper towel (*n* = 6).

CONCLUSION

A simple and inexpensive procedure for creating µPADs via wax dipping has been successfully developed. Because of the nature of the wax dipping process, several wax-based products, including beeswax, microwax, microparaffin wax and polyethylene wax, were mixed and melted to create distinctive textures for the hydrophobic areas on the µPADs and to improve the thermal stability of these devices, which is necessary in the high temperature environments found in many developing countries in tropical regions. The lab-on-paper device fabricated using beeswax containing PE was found to resist temperatures of up to 45 °C for 15 minutes. Lab-on-paper devicesfabricated using wax dipping are simple, rapid, easy-to-use, inexpensive, and disposable, and they can be used to simultaneously detect biomarkers in human specimens on one device using only one drop of whole blood or plasma. The device has the potential for rapid clinical diagnostics in the case of emergencies or in the event that no laboratory equipment is available, particularly in rural areas and developing countries.

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