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Novel, simple and low-cost alternative method for fabrication of paper-based microfluidics by wax dipping

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ABSTRACT

Paper-based microfluidic devices are an alternative technology for fabricating simple, low-cost, portable and disposable platforms for clinical diagnosis. Hereby, a novel wax dipping method for fabricating paper-based microfluidic devices (μPADs) is reported. The iron mould for wax dipping was created by a laser cutting technique. The designed pattern was transferred onto paper by dipping an assembly mould into melted wax. The optimal melting temperature and dipping time were investigated. The optimal melting temperature was in the range of 120–130 °C, and the optimal dipping time was 1 s. The whole fabrication process could be finished within 1 min without the use of complicated instruments or organic solvents. The smallest hydrophilic channel that could be created by the wax dipping method was 639 ± 7 μm in size. The reproducibility of the μPAD fabrication for hydrophilic channel width of the test zone and sample zone was 1.48% and 6.30%, respectively. To verify the performance of the μPAD, multiple colorimetric assays for simultaneous detection of glucose and protein in real samples were performed. An enzymatic assay and the bromocresol green (BCG) method were conducted on the paper device to determine the presence of glucose and protein in a test solution. The results of the assays were not significantly different from those of the conventional methods ($p > 0.05$, pair *t*-test and one-way ANOVA method). The wax dipping provides a new alternative method for fabricating lab-on-paper devices for multiple clinical diagnostics and will be very beneficial for developing countries.

1. Introduction

Lab-on-a-chip (LOC) devices have been developed to minimise the scale of laboratory tests. These LOC devices only need a small volume of the reagents and samples, therefore providing portable and disposable diagnostic devices [1,2]. However, the fabrication processes of LOC devices are quite complicated, as demonstrated by the need for mechanical components, such as pumps or valves, to control the flow of the solution within the microfluidic device.

Currently, paper tests or strip tests are widely used in clinical laboratories for diagnosing various diseases. The strip tests are utilised in several areas of healthcare, such as screening tests, self-monitoring by patients, treatment monitoring or preventive medicine. Recently, Whitesides's group has developed microfluidic paper-based analytical devices (μPADs) [3], also known as a lab-on-paper technology. The concept of a μPAD is to perform an experiment on a small piece of paper. Unlike the conventional strip test, the lab-on-paper devices can be configured for multiple tests or detection of several analytes simultaneously on one device [4]. Furthermore, quantitative measurement using a μPAD is feasible based on a variety of detection methods. Colorimetric assays on paper [5,6] are widely used to quantify the colour intensity of the test zone because it is easy to actualise and only requires simple equipment such as a digital camera, cell phone or scanner [4,7]. Moreover, a μPAD is able to perform several types of measurements, including electrochemical [8–11], transmittance [12], fluorescence and absorbance measurements [12,13]. According to WHO guidance, lab-on-paper devices are very promising for use as diagnostic tools in developing countries [4].

Currently, lab-on-paper devices have become an attractive technology for a number of research groups, resulting in the development of numerous methods for their fabrication. Various methods for fabrication of the μPAD have been proposed in the literature, including the following: photolithography [3,14], polydimethylsiloxane (PDMS) plotting [15], inkjet printing [16,17], cutting [18], plasma etching [19], wax printing [20–22] and wax screen-printing [23]. Photolithography was the first reported fabrication method, which involved the use of hydrophobic

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SU-8 photoresist and UV light to construct the hydrophobic and hydrophilic barriers on the paper [3]. This method can create a small barrier (200 μm width) and yield sharp resolution between the hydrophilic and the hydrophobic channels. However, the photolithography technique requires several organic solvents, which can damage the flexibility of the paper. In addition, photolithography requires expensive instrumentation and the fabrication process involves many complicated steps. The PDMS plotting method uses a desktop plotter and a hydrophobic polymer, namely PDMS, to create hydrophilic patterns on paper [15]. Although PDMS plotting does not destroy the flexibility of the paper, this method requires special preparation of PDMS diluted in hexanes [15]. The inkjet printing method involves removing a hydrophobic coating from the paper by using a modified an inkjet printer to print a solvent onto paper that has been coated with a hydrophobic polymer. The solvent melts the hydrophobic polymer, resulting in the formation of hydrophilic areas on the paper [16,17]. This method can create direct patterning on paper, which is a benefit for high mass production. Plasma etching is a method to remove a hydrophobic coating on paper by using plasma treatment [19]. However, the hydrophilic areas generated by both the inkjet printing and plasma etching methods are still exposed to solvents and polymers during the fabrication processes. In cutting method, a knife plotter is used to cut paper into designed microfluidic channels [18]. Nevertheless, the paper devices have to use tape to help support the paper structures, limiting the ability to produce variety of free-standing hydrophilic patterns [4]. Wax printing has several advantages such as fast and easy to produce, use commercially available printer and hotplate, and preserve native paper chemistry [20,22]. However, it is difficult to produce the exact designed patterns with high resolution due to the spread of the wax. Careful determination of wax spreading must be considered before production of the channels [4,22].

Common obstacles to fabricating lab-on-paper devices for most developing countries are the cost of the instruments used in the fabrication process, such as a spin coater, UV lithography system, and plasma cleaner. Although wax screen-printing, which requires only a hot plate for patterning wax onto paper, is economical and therefore promising for developing countries, it suffers from poor reproducibility [23]. Hence, a simple, rapid and cheap fabrication technique that also provides good resolution and repeatability needs to be developed.

This paper proposes a novel method for the fabrication of paper-based microfluidic devices by wax dipping. Wax is a material generally used worldwide because it is inexpensive and non-toxic. The wax dipping procedure requires only a hot plate for patterning hydrophobic and hydrophilic areas on Whatman No.1 paper. The fabrication of the μPAD is simple and only involves a single step. Moreover, the wax dipping method can create patterns on paper without using any chemical compounds, so that the hydrophilic area is not exposed to any solvents or polymers. To demonstrate its applicability to real world situations, we also employ the paper device for colorimetric assays for simultaneous detection of glucose and protein in real human samples.

2. Materials and methods

2.1. Materials and chemicals

Whatman No.1 filter paper was purchased from Whatman International, Ltd. (Maidstone, England). White Beeswax pellets were purchased from a stationary shop in Bangkok, Thailand. Glass slides were obtained from Sail Brand (Jiangsu, China). Iron moulds (1 mm thick) were made-to-order by a laser cutting shop in Bangkok. Permanent magnets were purchased from a local area shop. Other equipment that was purchased included a Canon digital camera (7.1 megapixels, Powershot A570 IS), an IKA® hotplate (C-MAG HS7, Wilmington, USA), and an Olympus Microscope (Olympus BX50, Tokyo, Japan).

D-(+)-Glucose, glucose oxidase (from *Aspergillus niger*-Type II), peroxidase (Type I from horseradish), potassium iodide, ethylenediaminetetraacetic acid disodium salt (EDTA), Brij™ 35 and bovine serum albumin were purchased from Sigma–Aldrich. Sodium hydroxide and succinic acid were purchased from Merck. Bromocresol green was supplied by BDH Chemicals. Glucose reagent (GLUCOSE liquicolor) and human control serum (Humatrol N and Humatrol P) were obtained from HUMAN (Wiesbaden, Germany). Accu-Chek for blood glucose monitoring was obtained from Roche Diagnostics. All chemicals were prepared in MilliQ water.

2.2. Wax dipping fabrication method

To create a mould for wax dipping, a local laser cutting shop cut an iron bar into the desired shape and size using a laser cutting technique. The price for cutting an iron mould was about $0.35 US per piece. An iron mould can be repeatedly used to produce numerous pieces of μPAD. In particular, based on our experience so far, more than 1000 pieces of μPAD have been fabricated from the same iron mould without affecting the resolution. For the wax dipping method, white Beeswax pellets were put in a beaker and heated until they melted using a hotplate. To ensure that the temperature was kept in the range of 120–130 °C, the temperature was monitored throughout the experiment by means of an electronic contact thermometer (IKA® ETS-D5). Whatman No.1 paper was cut into a 1.5 cm × 2.5 cm piece and placed onto a glass slide. Then, the iron mould was put onto the paper, and it was temporarily attached by means of magnetic force using a permanent magnet placed on the backside of the glass slide. Next, the assembly was dipped into a chamber of melted wax for 1 s. After the paper was cooled to room temperature, it was peeled off of the glass slide, and the iron mould was removed from the paper. The wax-dipping fabrication process for the μPAD is shown in Fig. 1. Then, the hydrophobic and hydrophilic areas of the μPAD were observed under a microscope (Fig. 2).

2.3. Applicability of the μPAD for clinical analysis

To evaluate the colorimetric assays on the μPAD, the paper device was designed to be a Y shape, which was composed of two test zones (circular shape, 3 mm width) for the simultaneous detection of glucose and protein. For the glucose assay, the reagent ratio was adopted from Self-Stik reagent strips (Chungdo Pharm. Co., LTD, Korea). A volume of 0.5 μL of a 10 mg mL^{-1} potassium iodide solution was spotted onto the paper test zone and allowed to dry for 5 min. Then, a 5 μL mixture of 451 U mL^{-1} glucose oxidase and 186 U mL^{-1} peroxidase was dropped on the same test zone. For protein detection, 0.5 μL of 10× bromocresol green (BCG) working reagent [24] was dropped on the other test zone. Then, the paper was allowed to dry at room temperature. To detect glucose and protein, the bottom side of the paper devices was dipped in sample solutions until colour developed at both test zones and could be observed by the naked eye. The colour of the test zones on the μPADs were captured by a digital camera, and then, the colour intensities were analysed using Adobe Photoshop CS2.

3. Results and discussions

3.1. μPAD made with the wax dipping method

The wax dipping method uses melted wax to coat a hydrophobic barrier onto paper while the hydrophilic channel is protected by an

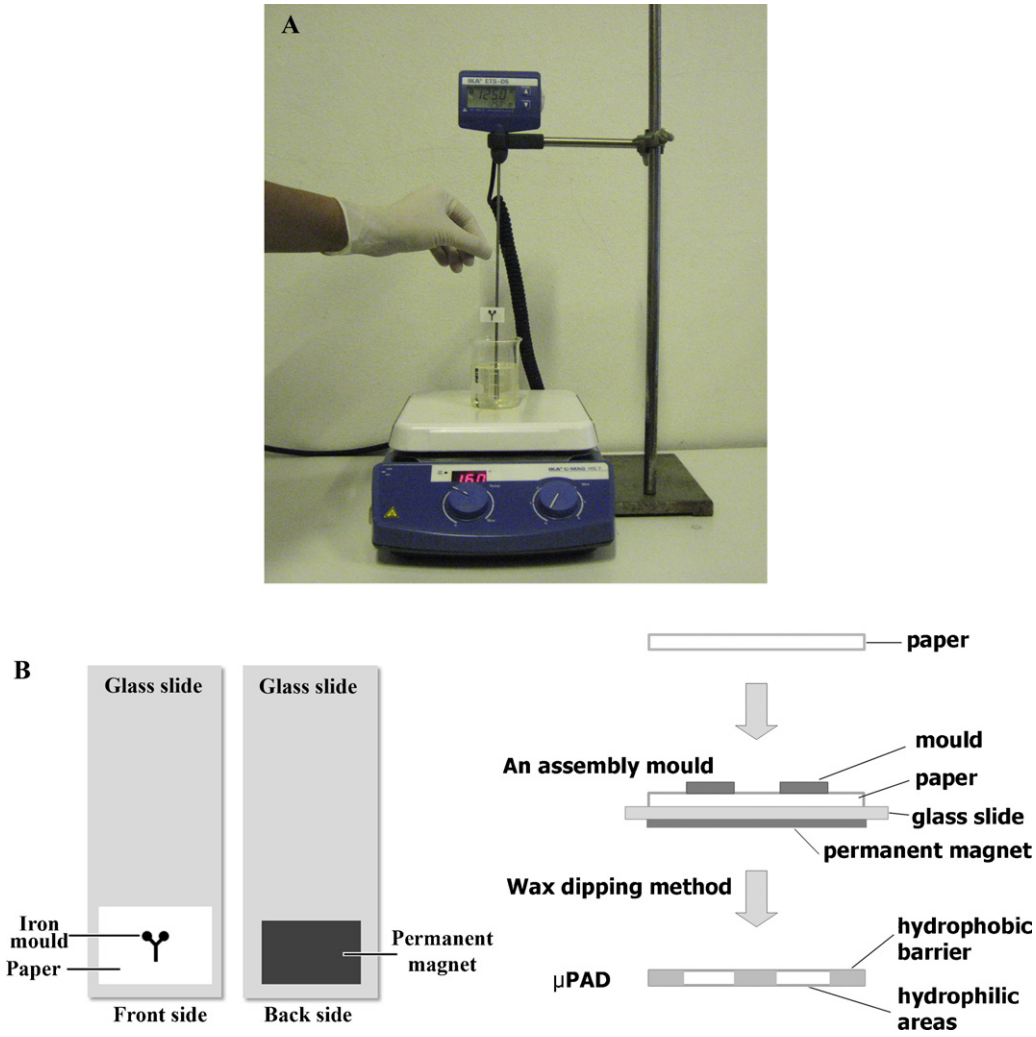

Fig. 1. Fabrication process of the μPAD using the wax dipping method: (A) simple wax dipping set-up system and (B) procedure for patterning paper by wax dipping in top view (left) and lateral view (right).

iron mould. When the paper was dipped into the melted wax, the melted wax penetrated into the membrane of the paper whereas the area obscured by the mould did not absorb the melted wax. Therefore, patterns of hydrophobic and hydrophilic areas were generated on paper. The fabricated pattern on the paper was observed by a microscope (Olympus BX50), as shown in Fig. 2A and B. It was clearly observed that the surface of the paper was changed as a result of the wax coating (Fig. 2A). The left side of the paper is the native surface, whereas the right side was coated with wax and completely turned into a hydrophobic surface. Fig. 2B indicates that coloured food dye could not percolate into the hydrophobic area because of the wax coating. The patterned paper fabricated by wax dipping could retain fluid within the hydrophilic channel; therefore, this area could be used for the reaction of a reagent and sample. Fig. 2C shows the hydrophobic property of our μPAD compared to the hydrophilic zone. The droplet of coloured food dye was apparently observed. It was not readily absorbed into the paper due to the hydrophobic surface of the paper.

3.2. Optimisation melting temperature and dipping time

The melting temperature and dipping time influenced the penetration of the melted wax into the paper and also affected the resolution between the hydrophobic and the hydrophilic areas. The optimal melting temperature and dipping time were investigated. The results demonstrated that dipping the paper into the melted wax when the temperature was lower than 120 °C for 1 s did not result in good resolution, in that the hydrophobic and hydrophilic areas were not completely separated. On the other hand, using a melting temperature above 130 °C resulted in excessive wax spreading into the paper. For the optimal dipping time experiment, the paper was dipped into the melted wax for varying amounts of time, from 1 to 20 s. It was determined that the optimal time for dipping the paper in the melted wax was only 1 s (just dipped and immediately lifted out). Dipping times longer than 1 s could not generate the patterns on the paper because of excessive spreading of the wax into the paper. In addition, using a high temperature (>130 °C) and long dipping time (>5 s) can boil the paper, causing it to separate from the glass slide. Therefore, the optimal melting temperature for wax dipping was 120–130 °C, and the optimal dipping time was 1 s (see supplementary data Table S-1). However, the temperature used for wax dipping in all subsequent experiments was at 125 °C.

Our wax dipping process is simpler and quicker in comparison to other wax printing methods because it only requires a dipping step, while wax printing needs both printing and heating steps. This wax dipping method takes less than 1 min to complete the whole process, whereas photolithography methods require at least 15 min [13] and wax printing methods use 5–10 min for prototyping the paper [20]. With consecutive dipping, the throughput of this

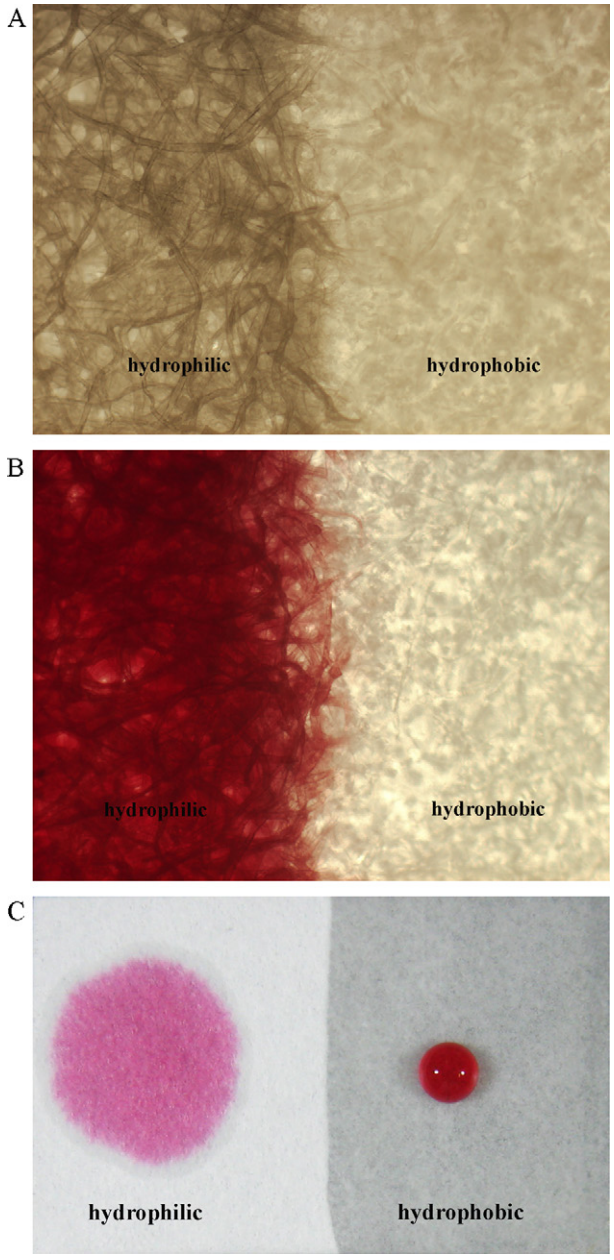

Fig. 2. Pictures of paper fabricated by wax dipping method: (A) hydrophobic and hydrophilic area captured under a microscope (40× magnification), (B) the hydrophilic zone soaked with food dye colour and (C) comparison of hydrophilic and hydrophobic area of the paper after applying a drop of coloured food dye.

method can be up to 90 pieces per hour. Moreover, wax printing methods require rather expensive printers and heating equipment, whereas this wax dipping method uses only a common hot plate or similar heating device.

Besides being a simple and rapid process for the fabrication of the μPAD, the proposed wax dipping protocol is also inexpensive and environmentally friendly. Every developing country can set up the wax dipping method for fabrication of μPADs. Beeswax is inexpensive and can be obtained worldwide, and the system set-up requires only a common hot plate, iron mould and permanent magnet. The total cost for a piece of μPAD fabrication by wax dipping is inexpensive at less than $0.05 US per piece. Some materials are reusable, such as the iron moulds, permanent magnets, and glass slides. From our point of view, the wax dipping method is a novel alternative technique to produce a simple and low-cost

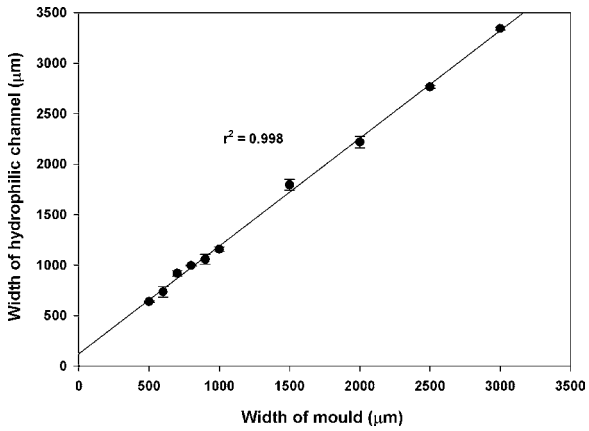

Fig. 3. Comparison of the width of the mould and the resulting hydrophilic channel on paper with the linear equation $W_C = 1.067\,W_M + 121.78$ ($r^2 = 0.998$).

lab-on-paper device, which is valuable for point-of-care testing, especially in developing countries.

3.3. Width of hydrophilic channel

The actual width of the hydrophilic channel after the fabrication process is dependent on the width of the iron mould used. To study the relationship between the widths of the hydrophilic channel and the widths of the initial mould, various sizes of moulds (500–3000 μm) were used to fabricate paper devices at the optimal melting temperature and dipping time. Unless otherwise stated, the dipping temperature was controlled at 125 °C and the dipping time was 1 s. The fabricated paper devices were imaged by a digital camera, and the dimensions of the channels were measured using ImageJ. Fig. 3 shows the width of the hydrophilic channel versus the width of the mould. The results show that the narrowest hydrophilic channel that the wax dipping method can generate using a 500 μm width mould was at 639 ± 7 μm ($n = 3$). Compared to other methods, the smallest hydrophilic channel that our proposed method can generate is on a similar size scale as the wax printing and wax screen-printing methods, in which the channels can be fabricated at 561 ± 45 μm [22] and 650 ± 71 μm [23], respectively. Even though the wax dipping method did not effectively produce hydrophilic channels as small as photolithography can (186 ± 13 μm) [14], the width obtained by wax dipping is acceptable to use in the paper-based microfluidic field. Furthermore, the relative standard deviation of the smallest hydrophilic channel using our proposed method (~1%) was found to be lower than other methods (~10%) [23]. According to the graph shown in Fig. 3, the resulting width of the hydrophilic channel can be calculated by using the linear equation $W_C = 1.067\,W_M + 121.78$ ($r^2 = 0.998$), where W_C is the width of the hydrophilic channel and W_M is the width of the mould.

3.4. Reproducibility of μPAD fabrication

Reproducibility is the variation arising when an experiment is repeated under the same conditions. To evaluate the reproducibility of the fabrication of the hydrophilic channels, 20 pieces of paper ($n = 20$) and a Y-shape mould composed of a 4 mm test zone and a 1 mm sample zone, were dipped in melted wax under the optimal conditions. Pictures of the paper devices were captured and used to measure the widths of the hydrophilic channel by using ImageJ. The relative standard deviation (RSD) of the widths of the obtained hydrophilic channels was calculated from the width measurements. Fig. 4 displays the reproducibility of the wax dipping fabrication for each piece of paper. The average width of

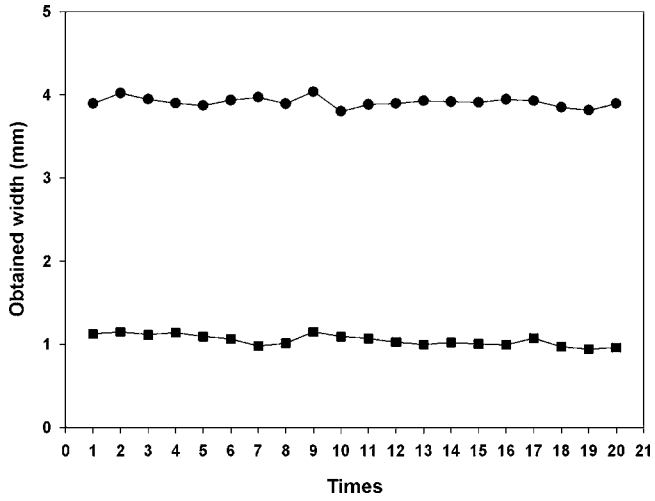

Fig. 4. Repeatability of the width of the hydrophilic channel in (■) the sample zone and (●) the test zone on the same device.

the hydrophilic test zone and sample zone were 3.91 ± 0.06 and 1.04 ± 0.07 mm, respectively. The RSDs for the width of the test zone and sample zone were 1.53 and 6.73%, respectively, which indicates our wax dipping method has good reproducibility. The proposed wax dipping method is more reproducible than the wax screen-printing method, which has a RSD around 11% [23]. The wax screen-printing method suffers from poor reproducibility because it is difficult to control the force applied to the solid wax to push it through the mesh screen. However, in our case, to fabricate the desired μPAD, the melting temperature and dipping time must be perfectly controlled in the optimal range throughout the process, as both factors affect the penetration of the wax into the paper.

3.5. Applications

The proposed μPADs fabricated by wax dipping were investigated for their applicability for the detection of glucose and protein in real samples based on colorimetric assays. Detail of the size and shape of an iron mould used in the experiments display in Fig. 5A. A 5 μL volume of 10× BCG working reagent or glucose reagent was spotted onto the separate detection zones of the μPAD device. After the reagents were allowed to dry for 10 min at room temperature, the bottom end of the μPAD device was dipped for 1 min into either a standard solution of glucose of varying concentration (0–1000 mg dL^{-1}), a standard solution of BSA of varying concentration (0–10 g dL^{-1}) or a sample solution.

Then, the colour intensity was allowed to develop from colourless to a strong blue or yellow for the protein and glucose reactions, respectively. Images of the colorimetric reaction of protein and glucose are shown in Fig. 5B. To measure the colour intensity, Adobe Photoshop CS2 was used to convert the images from RGB into grey scale format before analysis. A calibration curve was plotted for glucose or protein concentration versus colour intensity. The calibration curves are shown in Fig. 6A and B. The linear range for the glucose assay was 0–500 mg dL^{-1} (r^2 = 0.989) and 0–6 g dL^{-1} for the protein assay (r^2 = 0.990). The range of glucose concentrations that is linear for the assay in our proposed method is broader than for the conventional method based on the glucose oxidase spectrophotometric method, in which the linear range of GLUCOSE liquicolor is only up to 400 mg dL^{-1}. Similarly, the assay range for protein detection in our method is also better than the conventional BCG method, which is only linear up to 5 g dL^{-1}. Both results demonstrate that the wax dipping method for μPAD fabrication can be potentially applied for detection of glucose and protein

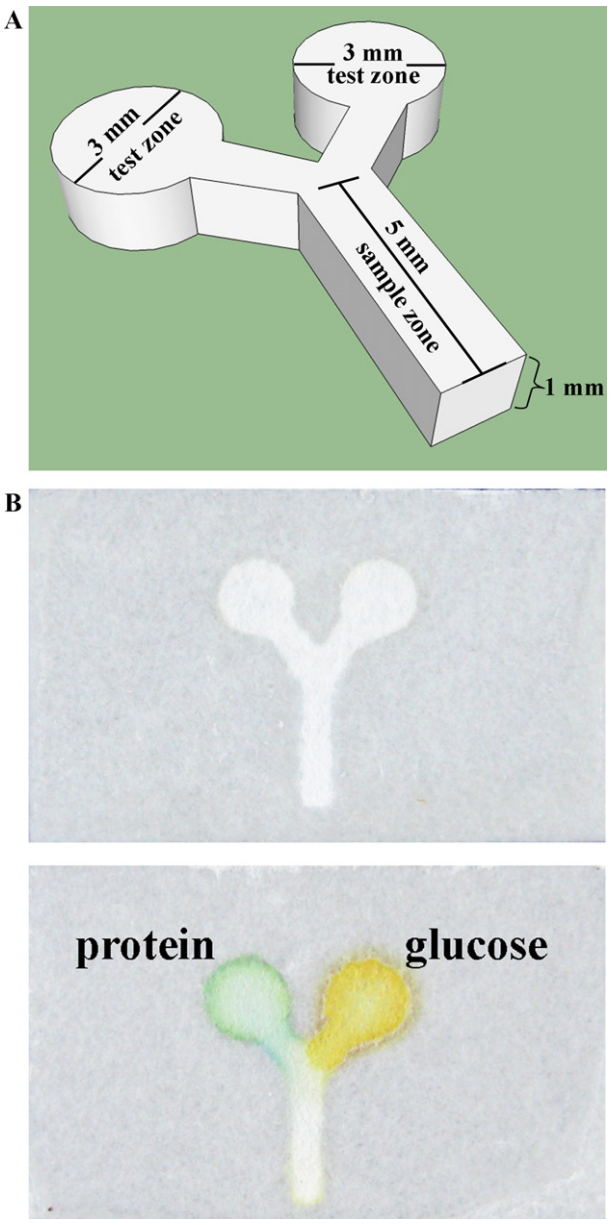

Fig. 5. Paper based microfluidic device fabricated by the wax dipping method used for colorimetric applications: (A) detail of the size and shape of an iron mould and (B) paper device after fabrication by the wax dipping method (top) and after use for the detection of protein and glucose (bottom).

simultaneously at levels that are clinically significant. Subsequently, the μPAD was utilised for quantitative detection of glucose and protein in real samples, and the results were compared to the conventional methods. Two samples of control serum with different levels of protein and glucose were tested by the wax dipped μPAD and conventional methods. The comparison of the results demonstrated that both methods were rather similar. When analysed by a paired *t*-test, no significant difference was found between the two methods at a 95% confidence interval. In addition, the μPAD was also used to measure the glucose level from real plasma samples, and the results were compared with both conventional enzymatic glucose oxidase and Accu-Check point-of-care-testing methods. The analysis of the results using one-way ANOVA demonstrated that the concentration of glucose in the plasma as determined by the three methods was not significantly different ($p > 0.05$) (Table 1). It can therefore be concluded that

Table 1
Determination of glucose and protein in real samples.

Control serum sample ($n = 3$)	Glucose (mg/dL)			Protein (g/dL)		p-Value
	Wax dipping μPAD	Conventional method (glucose oxidase)	Accu-Check	Wax dipping μPAD	Conventional BCG method	
Control N	94.4 ± 7.9	94.7 ± 1.5	nd	4.08 ± 0.51	4.10 ± 0.08	0.956[a]
Control P	229.7 ± 5.1	223.3 ± 11.9	nd	5.31 ± 0.35	5.81 ± 0.18	0.209[a]
Fasting blood glucose	89.7 ± 3.7	93.6 ± 0.6	86.5 ± 0.7	nd	nd	0.132[b]
1 h postprandial	136.2 ± 5.8	138.0 ± 4.6	137.0 ± 1.4	nd	nd	0.919[b]

nd = not determined.
[a] Results of paired t-test.
[b] Results of one-way ANOVA, $p < 0.05$ significance level.

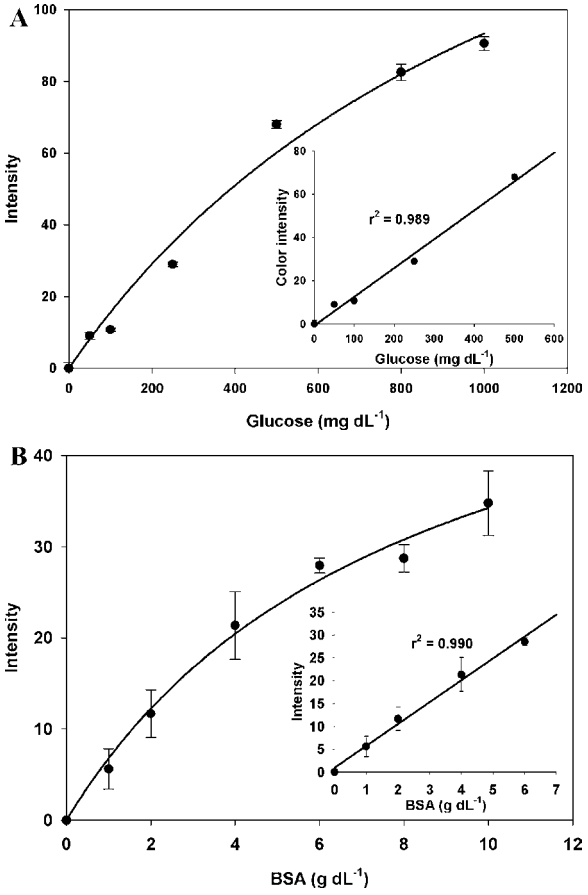

Fig. 6. The μPAD for colorimetric assay (A) standard curve of glucose ($r^2 = 0.989$) and (B) standard curve of protein ($r^2 = 0.990$) by using grey scale mode of Adobe Photoshop program.

the μPAD fabricated by the wax dipping method can be used for quantitative assays in the human samples.

4. Conclusions

The wax dipping method is a simple, rapid, and inexpensive method for fabrication of μPADs. Other advantages of this pioneering method are that there is no requirement for complicated and expensive instruments or organic solvents. Therefore, this technique provides an alternative and inexpensive platform for fabrication of clinical diagnostic devices in developing countries. A single dipping step can create microfluidic channels on paper within 1 min. Good resolution of the hydrophilic channel of the μPAD was obtained (%CV ~2–7%). Additionally, the width of the hydrophilic channel was highly correlated with the width of the designed mould, so the exact size of the μPAD can be predicted from the initial mould used. The crucial parameters to determine the resolution of the μPAD were the melting temperature of the wax and the dipping time. The smallest hydrophilic channel that can be fabricated using this proposed method measures about 639 ± 7 μm in width, which is sufficient for fabrication of the microfluidic paper based assay. Multiple colorimetric assays can be simultaneously performed on the μPAD, and the results revealed the ability to analyse glucose and protein in real samples. The wax dipping procedure preserves the native paper surface, therefore this technique does not face the problem of interference from residues remaining in the hydrophilic channel.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.08.024.

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