Local photo-polymer deposition-assisted fabrication of multilayer paper-based devices

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Abstract

In this paper, we report on the use of a local acrylate-based negative photo-polymer deposition technique for the fabrication of 3D microfluidic paper-based analytical devices (3D-μPADs) where the sample flows in both the lateral and vertical directions through multiple stacked layers of porous materials. A simple and inexpensive manufacturing method was used, which is based on the local deposition of a photo-polymer (deposition speed 30 mm/s) on a porous cellulose paper substrate followed by the subsequent exposure (scanning speed 30 mm/s) to a laser source (fibre coupled continuous wave at 405 nm with maximum power of 60 mW), to stack four layers of cellulose paper and make 3D multilayer μPADs. With this technique, we provide a pathway to eliminate the limitations that other reported methods have during the fabrication of μPADs such as the need for multiple sophisticated alignments between adjoining layers and the use of additional tools to ensure adequate contact between the layers. In this study, we demonstrate the usefulness of our four-layer 3D-μPAD for simultaneous detection of three analytes, namely BSA, glucose, nitrite spiked in artificial urine and also the pH of the tested sample, through single step colorimetric assays with the limit of detection found at 0.4 mg/ml for BSA, 14.5 μg/ml for glucose and 2.5 μg/ml for nitrite. Our 3D-μPAD fabrication methodology can also be adapted in more complex analytical assays where multiple steps are needed for applications in point-of-care diagnostics.

Keywords: 3D multilayer device; Multiplexed detection; Photo-polymer; Laser patterning method; Diagnostics; Paper-based device

1. Introduction

Public health is of universal importance and to that end, there is a constant research effort to create novel diagnostic tools world-wide [1,2]. According to the World Health Organization, a diagnostic sensor designed for use at the point-of-care in under resourced settings has to be affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free and deliverable to those who need it [3]. Among the different substrates, such as silicon, polymer or glass, which have been used to manufacture diagnostic devices, paper is a promising alternative due to its inherent characteristics. Paper is a cost-effective material composed of a network of hydrophilic cellulose fibres, and has a porous structure that allows the passive transport of liquids via capillary action [4]. Furthermore, paper is lightweight, can be found in different forms and properties, and is biocompatible and disposable via incineration [5]. The concept of paper-based analytical devices (μPADs) was first proposed from the Whiteside's group back in 2007 and has been developed for a range of analytical assays such as the detection of proteins [6], heavy metals [7,8], pesticides [9] and food-borne pathogens [10]. Such devices have the capability to obtain, control and process small volumes of complex fluids with efficiency and speed and the miniaturization of the device further reduces the detection times and the volumes of the reagents that are used [11].

In the last few years, a variety of methods to develop 2D-μPADs have been widely reported. Some of these include laser treatment [12], photolithography [13], wax printing [14] and inkjet printing [15] and have been shown to be used for various applications such as detection of biomarkers i.e. lactate, glutamate, uric acid and nitrite using different colour indicators in each case. Compared with 2D-μPADs, three-dimensional (3D) μPADs have also been widely studied due to some unique advantages they offer for certain applications, e.g. multiplexed assay and assays involve multiple steps. Firstly, in 2D-μPADs, the spatial distribution of samples is limited when compared with 3D-μPADs where the flow and delivery of fluids is enabled in all three dimensions. As a result, for 3D-μPADs the device footprint is reduced and also additional fluidic channels can be incorporated for enabling multiplexed testing of a large number of analytes with the same device footprint of a 2D device [16]. All of this leads to a reduced detection time and a reduced sample volume [17]. Despite the progress in the development of fluid flow control using different methods for more efficient performance of μPADs [18], it has been reported that 3D-μPADs exhibit better analytical performance when compared to lateral flow devices with sample flow limited in one direction [19]. In the case of 3D-μPADs, reagents can be stored within the middle layers of the device and furthermore as fluids transport within these layers the evaporation is minimized [20]. Additionally, using 3D-μPADs we can overcome the limitation of only being able to implement single step assays, as one can perform complex assays where mixing of different compounds is needed (e.g. ELISA) [21]. Furthermore, 3D-μPADs can eliminate any contamination of the sample as it can be largely transported within the in-between paper layers by creating appropriate fluidic pathways. Finally, it also helps prevent any cross-contamination between multiple samples thus enabling simultaneous testing of different assays independently within the same device.

3D-μPADs have therefore gained much attention of the research community in the last few years and many groups are working towards the development of these platforms. Reported fabrication methods include the development of 3D origami devices, which involve folding of the paper [22,23] and the use

of a stack of pre-patterned papers [24]. The origami-based methods, while ingenious, require sequential steps for assembly and alignment of the layers of paper, which might be difficult or incorrectly performed by untrained individuals. The most important requirement during the fabrication of 3D-µPADs is the need for sufficient contact between the multiple layers of the device. In order to assemble the pre-patterned paper layers and create a 3D-µPAD, a double-sided tape [25] or spray adhesive [26] is often used. Additional processes to ensure contact between the layers include the use of clamps [22,27] or even holding the device between the thumb and index fingers [28]. An alternative method reports the formation of 3D structures directly on the paper layer, a method that sorts out any contact issues but lacks simplicity as it requires double-sided wax printing and an extra aligning step during the fabrication process [29]. As described, each of these aforementioned techniques require external/additional equipment (e.g. clamps, tapes) and further fabrication steps, two factors that increase the complexity of the design. Additionally, the method of stacking the individual pre-patterned layers of paper requires an extra aligning step, which is a critical parameter for the performance of the final device.

In our previous reported method we used a laser direct writing technique to create 3D-µPADs [30]. That method not only involved additional fabrication steps such as polymer soaking and solvent-based developing of the paper device but was also limited in terms of the maximum number of layers which can be bonded together. In contrast, this paper reports the use of a simpler and faster fabrication technique, which enables the stacking of several layers of porous materials to create 3D-µPADs and exceeds the maximum number of the three layers that our previous method had.

We then demonstrate the use of this technique to produce a 3D-µPAD for implementation of single step colorimetric assays for the detection of three analytes, namely BSA, glucose and nitrite and the pH of the tested artificial urine sample. The choice of the model analytes was made because of the following - these analytes are commonly used in diagnostics (e.g. urine reagent strip testing) for monitoring or screening for kidney function, acid-base balance and urinary tract functions. BSA is the model protein for human albumin in urine and is studied extensively because it has similar composition [32] (e.g. molecular weight ~66 kDa) to the human protein. Elevated levels of albumin in urine corresponds to very low levels in blood and this is an indication of kidney damage (hypoalbuminemia). Elevated levels of glucose in urine is also an indication of underlying health conditions. In the case of people having diabetes [33] they can develop chronic complications such as kidney problems, which can lead to the excretion of glucose into the urine (glycosuria). Apart from the measurement of albumin and glucose levels in urine, the measurement of nitrite is equally important and very common in urinalysis. The presence of nitrites in urine can be an indication of bacterial infection (most commonly Escherichia coli) in the urinary tract [34], which if left untreated can lead to kidney failure or even sepsis. Finally yet importantly, pH measures how acidic or alkaline a person's urine is. Abnormal levels are associated with medical conditions such as kidney stones or urinary tract infections.

2. Experimental Section

2.1 Experimental setup and materials

The laser used for the polymerization of the photo-polymer was a fibre-coupled continuous wave (c.w.) diode laser (Cobolt MLD, Cobolt AB Sweden) operating at 405 nm with a maximum output power of 60 mW. As previously mentioned, the photo-polymer is locally deposited onto the paper substrate using a PICO® Pμlse™ dispenser platform from Nordson EFD, UK, which deposits micro-droplets through a tip with diameter 100 μm with volumes as small as 0.5 nL at a frequency at 100 Hz. The porous paper used to make the multilayer 3D-µPADs is Whatman™ grade 1 qualitative filter paper (cellulose) with nominal thickness 180 μm and pore size 11 μm from GE Healthcare. The substrate where the reagents for the assay are pre-deposited is glass fiber with 355 μm in thickness from GE Healthcare. The acrylate-based negative photo-polymer that we used in the device fabrication work was DeSolite® 3471-3-14 from DSM Desotech, Inc., USA. The performance of our paper-based 3D-µPADs was tested with single step colorimetric assays for the detection of BSA, glucose, nitrite and pH. For the BSA assay the reagents used were 49.2 mg/ml citrate buffer at 1.8 pH and 2.2 mg/ml tetrabromophenol blue (TBPB) in 95% ethanol solution (Sigma-Aldrich, 199311). The BSA protein (Sigma-Aldrich, A3059) which was used as the analyte for the BSA assay was prepared at a concentration of 50 mg/ml. The reagents used for the glucose assay were glucose oxidase/peroxidase solution (Sigma-Aldrich, G3660) in 5:1 ratio and 15 units of protein per ml in deionized water and o-Dianisidine dihydrochloride (Sigma-Aldrich, D2679)

with a concentration of 5 mg/ml in deionized water. D-(+)-glucose solution was used as the analyte for the glucose assay with a concentration of 1mg/ml in 0.1% benzoic acid (Sigma-Aldrich, G3285). For the detection of nitrite, we prepared Griess reagent by dissolving in deionized water 8.63 mg/ml sulfanilamide (Sigma-Aldrich, S9251), 63.3 mg/ml citric acid (Sigma-Aldrich, 251275) and 2.56 mg/ml of N-(1-naphthyl) ethylenediamine dihydrochloride (Sigma-Aldrich, 222488). We prepared sodium nitrite (Sigma-Aldrich, 237213) at a concentration of 0.68 mg/ml as the analyte for testing of this assay. Finally, for the pH testing we used bromothymol blue (Sigma-Aldrich, 114413) as an indicator and a red chemical dye (Allura Red AC, Sigma Aldrich) diluted in deionized water at a concentration of 0.5 mg/ml, to visualize the progression of the sample throughout the layers of the multilayer devices.

2.2 Methods and procedure

Our patterning method used in creating polymeric structures within the porous paper substrates and for the creation of the 3D-μPADs is based on the local deposition of an acrylate-based negative photo-polymer at locations pre-defined by the device design. The schematic in Figure 1 illustrates the setup used for the manufacture of our 3D-μPADs.

Figure 1

Figure 1 Schematic of the local photo-polymer deposition setup. The photo-polymer is locally deposited on top of a porous substrate and subsequently exposed to a c.w. laser source operating at 405 nm to create solid polymeric structures. In the dashed line box, we represent a cross-sectional image of the porous substrate in which the polymerized photo-polymer has extended throughout its thickness.

First, a dispensing system delivers the photo-polymer on top of a porous substrate at locations user-defined by a computer. Next, a laser source (spot size ~5 mm and fluence 40 mJ/cm^2) controlled by the same design program follows the same pattern and, illuminates the polymer inducing photo-polymerization leading to the creation of solid polymeric structures extending throughout the thickness of the substrate. A delay of 3 minutes between the deposition and photo-polymerisation steps was introduced to ensure that the photo-polymer had penetrated throughout the thickness of the paper (~ 180 μm). After this time delay, the photo-polymer had spread laterally to a width of ~2 mm, which defined the minimum width of the polymeric line that we can create with our patterning method for this specific substrate. The time required to make a single 3D-μPAD depends on the time required (3 minutes) for the photo-polymer to penetrate throughout a paper substrate. However, one of the advantages of the local photo-polymer deposition method is that we could fabricate six 3D-μPADs in a single cellulose paper A4 sheet within a period of 3 minutes. Considering that it required 12 minutes to pattern and stack the six, four-layer devices, we deduced that the time required to make a single four-layer 3D-μPAD does not exceed 2 minutes.

The photo-polymerization process is affected by the scanning speed of the laser and therefore the laser fluence. The fabrication conditions of the photo-polymer deposition speed, the laser scanning speed and the time delay between these two steps that we have used for the device processing are sufficient to fully polymerize the photo-polymer and create solid polymeric walls that define the boundaries of the flow paths within our devices. Any residual un-polymerized material that remains within these walls would result in sideways leakage of the fluids through them, and this is not the case as we see in either of the figures 4-6. Use of these fabrication conditions lead to the formation of fully solidified walls required to guide the flow of liquids without any leakage that would be detrimental to the performance of the device.

This patterning method was also used to stack several layers of the same (grade 1) porous material and create our multilayer 3D-μPADs. Figure 2 depicts the various steps involved in the fabrication of a two-layer 3D-μPAD. First, a photo-polymer pattern as required by the device geometry is locally deposited on top of a porous substrate. The photo-polymer is allowed to spread throughout its thickness (step 1) before exposure to the laser source (step 2). Next, the photo-polymer is deposited again (step 3) over the same pattern and a second paper substrate is placed (step 4) over the top. In order to facilitate complete contact between the two adjacent layers and therefore ensure proper flow of the sample between the layers, a contact pad of the same cellulose material is added between the two paper layers only at the

intersection points where the sample flows happens, from one layer into the other. The photo-polymer pattern is added again on top of the second layer (step 5), followed by exposure from the laser source to assist with the bonding of the two layers (step 6). This paper patterning technique can easily be extended to stack several layers of porous materials and create a multilayer device where the fluids are able to flow within the multiple layers of the user-designed device.

Figure 2

Figure 2 Schematic of the fabrication process that is used to stack two layers of paper.

In order to create 3D-μPADs where the samples travel in lateral and vertical directions and through multiple, stacked layers, as suggested above, we then used the described patterning technique to produce a multilayer device that implements four independent single-step colorimetric assays. Figure 3 shows the schematic of such a multilayer device that was fabricated to allow for single-step colorimetric assay based detection of BSA, glucose, nitrite and the pH. It consists of four different layers patterned and stacked using our method described via Figure 1 and Figure 2. In each layer, the sample can only flow in the hydrophilic areas, which are demarcated by the photo-polymer patterns preventing the flow into undesired locations. The top layer has an inlet port for introduction of the sample and the bottom layer has four rectangular readout zones designed to visualize the outcome of the colorimetric assays. The two middle layers allow distribution of the sample homogeneously into the four detection areas. To assist with the flow of the sample throughout the device, from the top layer all through to the bottom layer, contact pads (represented as a blue square in Figure 3) of a hydrophilic material were placed at the intersection of the channels between the adjoining layers. Cellulose paper was used for contacting the top three layers, whereas, glass fibre was used as the hydrophilic contact pad material between the third and the bottom layer containing the four detection zones. The glass fibre not only allowed proper flow of the sample between the third and the bottom layer, but also served as pads for storage of reagents required for each of the different assays. The reagents were deposited (and dried at room temperature) on the glass fibre pads prior to their addition between the two final layers of our device. The choice of glass fibre was due to its known attribute of effective release of the reagents on rehydration from an incoming flow of a liquid sample. As shown in Figure 3 red coloured lines indicate the direction of the flow of the sample from the top into the middle layers and then into the bottom layer where the outcome of the assays would be visualized.

Figure 3

Figure 3 Schematic of the 3D device used to make the single step colorimetric assays.

3   Results and discussion

3.1. Stacking method for the fabrication of multilayer devices

In order to evaluate the performance of our local deposition-assisted paper-stacking method, we next tested our devices using a red chemical dye that allowed us to visualise the fluid flow through the different layers. The results for an example are as shown in Figure 4. The dimensions of the squares (shown in Figure 4) were designed to be 5×5 mm^2 and the photo-polymer walls that defined those squares had a width of ~2 mm (the corresponding photo-polymer deposition speed was 30 mm/s). The laser exposure of the photo-polymer was at the same speed of 30 mm/s. To ensure the creation of solid photo-polymer structures that guide and hold the liquid sample the same protocol for deposition, time delay, and photo-polymerization was used to create the structures within each of the paper layers. The laser power was set at 60 mW and had a spot size at 5 mm, which is larger than the width of the photo-polymer line and these ensured the complete curing of the photo-polymer. Figure 4 presents the results of different multilayer paper devices where we have successfully stacked two, three, four and five layers of paper following the same procedure each time and tested the flow within them with 20 μL of red dye. We followed the same

process to produce six devices for each of the four different multilayer cases and figure 4 shows images of these four different multilayer devices with 2-5 layers respectively. The results were consistent for each one of them. As can be clearly seen the red dye which is deposited from the top face of the device, flows through the successive layers of paper and ends up at the bottom face of the device. In order to visualize that the red dye flown through each of the different layers of our stack, a cross-sectional image was taken, which shows that in each case the red dye has successfully travelled through all the layers.

Figure 4

Figure 4 Multilayer paper device tested with a red chemical dye.

3.2 3D paper-based device

In these sections, we explore the capability of our patterning method to create 3D multilayer devices that can detect multiple analytes with a liquid sample – artificial urine. The fabrication parameters (photo-polymer deposition speed, time delay, scanning speed of the laser and the laser fluence) remained unaltered for fabrication of these devices. Figure 5 shows one such 3D device designed according to the schematic of Figure 3 where four layers of cellulose paper are patterned and stacked together to form a 20×20 mm^2 square device. The dimensions of the channels in each layer are as listed in Table 1. The performance of the 3D device was similarly evaluated using the same red dye; 35 µL of the dye was pipetted into the inlet point and the result was observed by viewing the bottom face of the device. The red dye provided an aid to visualize the progression of fluids through the multiple layers of this design and the volume of 35 µL is the least sample volume that is needed in order for it to flow through the successive layers, soak completely the middle paper layers, and end up at the bottom face of the device (figure 5).

Figure 5

Figure 5 3D multilayer paper-based device tested with a red dye. Left image: Top face of the device where the red dye is introduced. Right image: Bottom face of the device where we visualize the outcome of the testing process.

Table 1

Table 1 Dimensions of the channels within each layer of the 3D multilayer device.

We next evaluated the usefulness of our methodology by fabricating and testing the performance of devices with geometries (shown in Figure 3) identical to those above (four layers with two middle layers) for detection of three common analytes presented in urine. For this testing, artificial urine samples spiked with the three chosen biomarkers, BSA, glucose and nitrite were used, and the detection of each of these was through a colour development in individual detection zones within the bottom layer of the device. The intensity of the colour produced is also an indication of the concentration of the biomarker in the artificial urine sample and was therefore use to further not just detect, but also quantify the levels of the different markers in our samples. For the BSA the colour change within the detection zone should be from yellow to blue-green, for glucose the change should be from brown to pink, and for nitrite, the change should be from light pink to a darker reddish colour. In case of pH the colour changes from yellow (6.0) to green (7.6) for the corresponding pH values. As described earlier, the different reagents were pre-deposited and dried on the glass fibre pad that was placed between the bottom two layers of the device. The reagents that were pre-deposited in the devices for the different assays were as follows: For the BSA assay we used 1 µL of TBPB, for the glucose assay we used 2 µL of glucose oxidase/peroxidase solution, for the nitrite assay we used 1 µL of the Griess reagent and for the pH measurement we used 2 µL of bromothymol blue.

Figure 6 shows the bottom face of our 3D-µPADs at different times after 50 µL of the artificial urine sample was introduced from the top face of each device. The purpose of increasing the sample volume

from 35 µL to 50 µL for actual tests, is for the acquisition of a better sensitivity during the measurement of the tested analytes. From the results presented in figure 6, we know that the volume of 50 µL is sufficient for the sample to travel through all the layers of the 3D-µPAD without overloading the device and producing any sideways leakage of the sample. Once the sample rehydrates and reacts with the reagents stored in the in-between glass fibre pads it then carriers on to the detection zones to produce a corresponding colour change within the different detection zones. We performed a detailed study testing several devices with known samples concentration across a range of 0.3 mg/ml to 18.2 mg/ml for BSA, 7.3 µg/ml to 363 µg/ml for glucose and a range of 0.6 µg/ml to 62.6 µg/ml for nitrite. As can be clearly seen in Figure 6 there is a gradient colour change of the intensity for each of the analytes as their concentrations change from a lower to a higher value. The higher the concentration of the analyte in the sample, the more increased is the colour intensity in the detection zone.

Figure 6

Figure 6 Images of the bottom face of the four-layer 3D µPADs used to detect three analytes in artificial urine- BSA, glucose and nitrite, and measure of sample's pH. Images in (A) are for different concentrations of the analytes 1 minute after the assay. Images in (B) are for the different concentrations of the analytes 10 minutes after the assay.

For our 3D paper devices, the limit of detection (LOD) for the three analytes we chose to measure were found to be 0.4 mg/ml for BSA, 14.5 µg/ml for glucose and 2.5 µg/ml for nitrite. For the calculation of these values we used the following equations:

$$LOD = LOB + 3 \times SD_{\text{Lowest concentration sample}}$$

$$LOB = \langle \text{Blank} \rangle + 1.645 \times SD_{\text{Blank}}$$

LOB refers to the limit of the blank, blank refers to the devices tested with a sample containing no analyte and SD is the standard deviation.

In the case of BSA, as the value of the limit of detection of our 3D µPADs is 0.4 mg/ml, this allows the use of such a device for the detection of nephrotic syndrome (protein>2.32 mg/ml), subnephrotic range proteinuria (0.66<protein<2.32 mg/ml,) and tubular proteinuria (protein< 0.66 mg/ml). Additionally, the LOD of 14.5 µg/ml for glucose suggest its usefulness for the practical application of detecting elevated levels of glucose in urine (glucose levels greater than 250 µg/ml is an indication of diabetes). Last but not least, our reported device is capable of detecting nitrites in urine as their presence is an indication of a urinary tract infection.

The colour intensities on the test zones for each device were analysed using Adobe Photoshop. After using a scanner to take photos of the tested devices, the RGB channel in the histogram function in Adobe Photoshop was used to measure the mean intensity value of the pixels for each one of the four detection zones. The colour intensity values of the control device (no analyte) was subtracted from the values for the devices tested with the analytes in order to obtain the actual signal intensity. For BSA and nitrite, we measured the signal 1 minute after the assay (Figure 6a). However, as the glucose assay requires a longer time to complete, ~10 minutes, the colour was allowed to develop over this period of time, and intensity measured thereafter (Figure 6b). Calibration curves based on the actual colour intensities is shown in Figure 7 and as can be clearly seen, for each of the three analytes, as the concentration of the analyte increases, the corresponding intensity of the colour produced also increases. Each measurement was repeated three times to estimate the error.

Figure 7

Figure 7 Calibration curves showing the colour intensity of (A) BSA, (B) glucose and (C) nitrite for their different concentrations in the artificial urine samples.

4   Conclusion

In this paper, we report the use of a simple, fast and inexpensive method to create 3D-µPADs using porous paper substrates. This technique is based on the local deposition of an acrylate-based negative photo-polymer followed by its exposure to a laser source to make multilayer paper devices. Unlike other reported methods, our patterning technique eliminates the need for any sophisticated alignment that may be required between the layers further also does not mandate the need for the use of external or additional equipment during the assembly of the device. The performance of our method was tested by stacking several layers of paper and making multilayer 3D paper-based devices, which were tested with a red dye that visualize that the sample flowed through the layers without blockage or leakage. Finally, we used the 3D-µPADs (with reagents, dried and stored within) to perform simple one-step colorimetric assays for the detection of BSA, glucose, nitrite and pH using artificial urine samples. Based on the results we observed a colour change for each one of the biomarkers relative to the concentration that we used in our sample. Additionally, the devices were shown to be used for a quantitative detection of the tested analytes. We believe that our paper patterning method that allows for the fabrication of 3D-µPADs is promising and presents important advantages when compared with other reported methods and can be further explored for use in more complex assays, where multiple sequential steps are needed.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

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