Fabrication of microtiter plate on paper using 96-well plates for wax stamping

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Abstract

Paper-based analytical devices have prominently emerged as a group of diagnostic tools with prospective to eliminate the expensive, time-consuming, and intricate analytical methodologies. Wax printing has been a dominant technique to fabricate hydrophobic patterns on paper for fluid control, but the discontinuation of commercial solid ink printers has begun a genesis of alternate wax patterning strategies. In this study, a simple and rapid fabrication methodology for realizing a 96-well microtiter plate on paper has been developed. The method involves the use of commercially available polystyrene microplates as a stamp for wax patterning. The technique further eradicates the requirement of customized stamps and the step of heating paper substrates for creating wax barriers. Thus, wax stamped paper microplates can be used for a wide range of bioanalytical tests maneuvering reduced generation of non-biodegradable waste, minimal reagent usage, and inexpensive readout strategies. The viability of the fabricated platform has been assessed by colorimetric detection of glutathione using 3,3′,5,5′-tetramethylbenzidine–H2O2 redox system. RGB analysis of the colorimetric response showed a linear concentration range from 0 to 90 µM ($R^2 = 0.989$) along with a detection limit of 28.375 µM.

Keywords Paper · Wax stamping · Microtiter plate · Colorimetric detection · Glutathione

1 Introduction

Paper-based analytical devices (PADs) have been drawing significant attention for detecting analytes in biomedical, chemical, agricultural, and environmental fields since their initial demonstration by the Whitesides group (Martinez et al. 2007). Its applicability in human lives has been consequential, ranging from rapid urine analysis to detecting SARS-CoV-2 during the recent pandemic, under which the whole world travailed (Tai et al. 2021; Fabiani et al. 2022; He et al. 2015). The advantages of portability, low-cost manufacturing, minimal reagent use, facile disposability, reduced analysis time, and compatibility with mass production make PADs less laborious, practical, and sustainable (Ning et al. 2022; Pinheiro et al. 2021; Mi et al. 2022). It redeems the ASSURED (affordable, sensitive, specific, user-friendly, robust, equipment-free, delivered) criteria unfolded by WHO for diagnostic devices (Davidson et al. 2021; Manisha et al. 2017). These unique characteristics thrive the potential of PAD-based diagnostics in enhancing healthcare delivery for the people residing in resource-poor settings (Urdea et al. 2006).

Paper substrates possess the benefits of availability, low-priced, capillary action, hydrophilicity, and compatibility with a broad range of fabrication and processing techniques. Besides, the perks of exhibiting large surface-to-volume ratios and white backgrounds allow exemplary opportunities for envisaging colorimetric tests (Nishat et al. 2021; Das et al. 2022). Many methods have been developed for fabricating PADs, such as wax printing (Ma et al. 2018; Tesfaye and Hussen 2022), laser printing (Ghosh et al. 2019), inkjet printing (Su et al. 2016), plotting (Lee et al. 2010), screen printing (Rusling 2018), stamping (Zhang et al. 2014), flexographic printing (Tasaengtong and Sameenoi 2020), photolithography (Olmos et al. 2019) and plasma treatment (Christoffersson and Mandenius 2019). Among these, techniques utilizing wax-based processing are the most convenient as it is easily available, inexpensive, biodegradable, facilitate quick fabrication, permeability to paper, and potential to
“use and discard” (Jiang et al. 2022; Zhong et al. 2012) Solid ink printers have been predominantly used for fabricating customized wax patterns on paper substrates. However, due to the unavailability of solid ink printers in recent times, researchers have explored alternate wax patterning strategies involving various novel methodologies. Garcia et al. (2014) utilized a customized metal stamp for fabricating hydrophobic patterns by stamping manually with an application of ca. 2 kg onto the metal stamp. Mathaweesansurn et al. (2020) reported a contact ink-stamping method utilizing a custom-designed rubber stamp for defining the hydrophobic patterns. Similar efforts have been laid by various research groups in the view of realizing PADs which are inexpensive and easily accessible to researchers with limited resources. (Curto et al. 2013; Araujo et al. 2021; Noviana et al. 2021).

Microplates are widely used to perform bio-analytical tests in laboratories for various biological applications (Mishra 2022). They are prominently used for their capability of allowing multiplexed testing via different signaling strategies. However, routine use of these microplates generates a lot of non-biodegradable wastes as they are generally made up of polystyrene. Contrary, using microplates on paper can considerably reduce non-biodegradable waste generation. Furthermore, colorimetric analysis on paper microplates opens up opportunities for alternate readout strategies, circumventing the requirement of expensive microplate readers (Koesdjojo et al. 2015). Colorimetric detection of glutathione (GSH) has been attracting a large amount of attention due to its substantial role in the functioning of the human body (Ni et al. 2015). Quantification of GSH using 3,3′,5,5′-tetramethylbenzidine–H2O2 redox system further employs the congeniality with other routinely adapted biological detection methodologies, such as enzyme-linked immunosorbent assay (ELISA) and nanoparticle-mediated peroxidase mimicking (Tang et al. 2021).

This work elucidates a simple, rapid, and facile 96-well microplate on paper fabrication technique using wax stamping. To the best of our knowledge, this is the first report describing the usage of commercially available 96-well polystyrene plate as a stamp for wax patterning on paper. The selection of stamps is not limited to unused microtiter plates; used plates can also be utilized for the same. Moreover, the fabrication process does not require any heat treatment of the paper substrate. Wax penetrates finely through the filter paper during the stamping process itself. This prevents the modification of color and functional groups present in the paper substrate. The functionality of the fabricated microtiter plate has been estimated by detecting concentrations of GSH using RGB analysis.

2 Materials and methods

The fabrication technique involves usage of commercially available 96-well polystyrene microtiter plates as a stamp and a hot plate for melting the paraffin wax. Whatman Grade 1 qualitative filter paper is used as the paper substrate. Plastic lamination sheets and a TEXET A4 laminator were used for lamination of the filter paper. A hot air oven was used for heating the stamp plate before stamping. PerkinElmer FT-IR spectrometer (Spectrum two) and Olympus BX 51 microscope were used to characterize the fabricated microtiter plates.

Potassium dichromate (K2Cr2O7) solution used as a colored liquid to evaluate the hydrophobic barrier of the plate was purchased from MERCK, USA. Sulfuric acid (H2SO4), hydrochloric acid (HCl), sodium hydroxide (NaOH), buffer solution (pH 4), PBS buffer solution (pH7), toluene (C6H5CH3), acetone (CH3COCH3), methanol (CH3OH), ethanol (CH3CH2OH) and propanol (CH3CH2CH2OH) were procured from Sigma-Aldrich/Merck, USA, for evaluating solvent compatibility of the plate. Paraffin wax granules were purchased from Krokio Products Pvt. Ltd., India. Horseradish peroxidase (HRP), 3′3′5′5′-tetramethylbenzidine (TMB) liquid substrate solution, and glutathione were procured from Sigma-Aldrich, USA for colorimetric detection of glutathione. HP Color Laser Jet Pro (MFP M177Fw) was used for scanning the paper microtiter plate after performing colorimetric detection on the plate.

The realization of paper microtiter plate was initiated with a process of lamination to provide support from the back. Two filter papers were kept inside a single lamination sheet for hot lamination so that both sides of each filter paper did not get laminated. After the lamination process, the edges of the laminated sheet were cut so that the individual parts can be separated and used for the wax stamping process. Thereafter, wax was heated using a hot plate kept at 130 °C and the microtiter plate stamp was heated at 100 °C for 10 min in an oven. The top surface of the heated plate was dipped in the melted wax for 10 s to maintain the amount of melted wax sticking to the stamp and stamped manually on the laminated filter paper. Stamping was done with an application of ca. 800 g weight until the wax penetrated thoroughly to form hydrophobic barriers. The schematic of the fabrication technique is depicted in Fig. 1. Various parameters, such as wax heating temperature, heating of the stamp plate, stamp weight, and stamp contact time, were optimized during the process.

To determine the functionality of the fabricated paper microtiter plate, a colorimetric test for the detection of GSH was carried out. In this context, a stock solution of
GSH (320 µM) was prepared in water and different concentrations of GSH were prepared by diluting the stock solution. For the detection of GSH, 200 µl of commercially available TMB substrate solution and 10 µl of HRP solution were mixed and kept in dark for 15 min. After that, 20 µl of the colored solution mixture of ox-TMB followed by 10 µl of GSH solution with different concentrations were sequentially dropped on the wells of the plate and was kept for 5 min and then scanned in the flat-bed scanner for capturing a 300 dpi digital image of the test plate. The colorimetric analysis was performed by reading the RGB values of the test wells using the freely available ImageJ software. The obtained values were correlated with the concentrations of GSH, which was further used for evaluating the limit of detection (LOD) and linear concentration range.

3 Results and discussion

3.1 Fabrication process

The fabrication of the paper microtiter plate was carried out by manually stamping melted wax onto the paper substrate using a 96-well microtiter plate. The stamping process was made free of operator-dependent external parameters by fixing a metal weight onto the stamp plate so that the pressure is exerted by the weight of the stamp (ca. 800 g) itself. It was observed that heating wax around its melting point and stamping the stamp without any heat treatment led to severe distortions in the formed pattern. The reason was the solidification of wax on the stamp plate due to the fast drop to temperatures below its melting point.
point. Hence, a study was conducted to optimize the heating conditions. The wax was heated at different temperatures of 70 °C, 90 °C, 110 °C, 130 °C and 150 °C and the stamp was heated at 100 °C in an oven for 10 min. The heating of the stamp allowed the melted wax to maintain its mobility until it penetrated through the paper substrate. Heating the wax to 130 °C generated a proper replica of the stamp plate on the laminated paper substrate. Furthermore, variations in stamp contact time were also studied for 15, 30 and 45 s by keeping other parameters constant. The contact time of 15 s did not allow the full penetration of the wax through the paper substrate whereas the same for 45 s initiate blocking of wells due to over deposition of the wax from the stamp to the laminated paper substrate. Therefore, a contact time of 30 s is considered an optimized incubation time which allows full penetration of the wax through the paper for fabricating a well-replicated microtiter plate on paper substrates. Optimization of the fabrication process is depicted in Fig. 2 based on wax melting temperature keeping the other parameters in the optimized condition. Thus-formed hydrophobic barriers were evaluated by adding 20 µl of the colored solution onto the realized wells.

Backside lamination of the paper substrate allows the resistance of fluid flow from the hydrophilic parts of the individual wells and also provides back support. A study was conducted to evaluate the effectiveness of the back support by comparing non-laminated, cold laminated, and hot laminated paper microtiter plates. In this context, 20 µl of colored solution was added to the laminated plate wells and observed after 30 min to estimate the wicking resistance. It was observed that cold lamination cannot seal the backside completely, while hot laminated plates can hold and resist the flow of liquid from the wells for several hours. Heating the wax at 130 °C led to easy penetration through the paper substrate, adhering to the plastic lamination sheet beneath. This adherence seals the gap between the paper substrate and the lamination sheet and prevents the wicking of liquid from the gap between the paper substrate and the back support. It is noteworthy to mention that increasing temperature substantially beyond 130 °C led to the loss of rigidity of the stamp after using it once. Henceforth, hot lamination of the paper substrate before stamping and heating the wax at 130 °C has been considered the optimized condition for preparing the paper microtiter plate, while the temperature of the oven used for heating the stamp plate before stamping is kept constant at 100 °C for 10 min. The stamp plate can undergo multiple stamping cycles; more than 30 microtiter paper plates have been fabricated using the same stamp plate. The wax that sticks onto the stamp plate after a stamping process gets removed during the next wax dipping step required for subsequent fabrications.

### 3.2 Characterization

Structural assessment of the fabricated paper microtiter plates has been done by measuring the diameters of each well. For each test well, measurements were taken by averaging two cross diameters for ensuring the shape. To estimate the efficiency of the fabrication process, a range of 10 nos. of fabricated plates has been considered. The average well diameters of the fabricated plates were plotted in Fig. 3b. The plates showed a variation from 6.984 to 6.989 mm in average well diameters. Apart from this, a comparison was drawn to understand the usefulness of heated stamps for the stamping process. The study was again conducted for 10 nos. of fabricated plates. It was found that without heat treatment, only around 46% working wells were formed on average whereas it was 100% for the heated stamp case (Fig. S3). The results of the study have been depicted in Fig. 3c. It is noteworthy to mention the criteria for considering a working well, which has been the formation of a proper hydrophobic barrier for resisting the fluid flow and the average well diameter falling in the above mentioned variation range.

The liquid holding capacity of the plate was evaluated by adding different amounts of colored liquid (10–100 µl) to the wells of the plate under ambient conditions (temperature—22–28 °C, relative humidity—75–80%). Tests were done to assess the effectiveness of the hydrophobic barriers by estimating the maximum holding capacity of the

![Fig. 2](image-url) Evaluation of the fabrication process at different wax melting temperature a at 70 °C wax is not deposit on the paper as the wax stuck to the stamp due to its high solidification nature at lower temperature b at 90 °C wax is not fully deposited on paper c at 110 °C wax is not fully penetrated through the paper d at 130 °C wax is fully penetrated through the paper and a well-replicated microtiter plate is formed on the paper e at 150 °C melted wax blocks the plate well due to higher mobility of the wax at higher melting temperature. Each of the scale bars is 1 cm
fabricated wells without any wicking from the hydrophobic regions. One column between the liquid-added columns was left blank to make wicking visible, in case it traverses the hydrophobic barrier of the paper microtiter plate. To detect any wicking from the hydrophobic regions, the plate was observed for up to 8 h in regular time intervals, then after 24 h (next day) and found that it can hold up to 100 µl of liquid. The columns with 10, 20 and 30 µl volumes of liquid dried in 1.5, 3.5 and 6 h respectively under ambient conditions. The remaining columns dried within 24 h. It was observed that there was no wicking for columns with volumes up to 50 µl of colored solution whereas there was slight wicking from wells with liquid volumes 50–70 µl. Beyond 70 µl, the wicking was comparably higher as observed after 24 h of drying in ambient conditions (Fig. S1). Solvent compatibility of the plate was studied by adding 30 µl of different solvents, such as H2SO4, HCl, NaOH, acidic buffer (pH-4), and PBS (pH-7.4), which showed compatibility with the hydrophobic barrier. However, xylene, toluene, acetone, and other organic solvents were found incompatible to the plate. For making the compatibility of solvents visible, after 3 h of adding the solvents, 5 µl of colored liquid was added to the wells (Fig. S2).

Optical microscopy has been used to visualize the microstructures of the paper microtiter plate. Figure 4 shows the various images of the components of the fabricated plate. The wells of the fabricated plate were found very similar to the plain filter paper structure and endorse the suitability of wax as a favorable material for the stamping process. Further, the holding capacity of the well entrusts the formation of a continuous hydrophobic barrier through the stamping process.

To examine the presence of wax in the wells of the fabricated paper microtiter plate, FTIR spectroscopy was used. The analysis of functional groups in different sections of the plate can be used to characterize the presence of wax (Fig. 5). The characteristic peaks of wells of fabricated paper were observed at 3300 cm\(^{-1}\), 2904 cm\(^{-1}\), 1636 cm\(^{-1}\),
and 1032 cm\(^{-1}\), which are assigned to the –OH stretching, –CH\(_2\) stretching, H–O–H stretching and C–O, C–C stretching, respectively, and are consistent with characteristic peaks for cellulose fiber. The characteristic peaks of wax-embedded paper at 2915 and 2848 cm\(^{-1}\) were observed due to the asymmetric and symmetric stretching of aliphatic hydrocarbon respectively. The in-plane vibrations of aliphatic hydrocarbon are located at 1417, 1464, and 1375 cm\(^{-1}\), while the peak due to the rocking of the same vibrational group is found at 720 cm\(^{-1}\). These characteristic peaks of wax were absent in the wells of the fabricated plate. Hence, the functional group characterization confirms the absence of wax in the wells of the paper microtiter plate.

### 3.3 Functional estimation

The functional estimation of the fabricated paper microtiter plate was evaluated by colorimetric detection of GSH. In this regard, different concentrations (0–120 µM) of GSH were added to different wells of the microtiter plate containing ox-TMB solutions. With the increase in the concentration curve. The error bars illustrate the standard deviations of three independent measurements with the average (\(n = 3\)) mean value.
of GSH, the blue color of the solutions decreased gradually, which resulted in a change in colors of the solutions from blue to light blue to colorless. The change in color with the concentration of GSH was correlated digitally using image processing via ImageJ software. The scanned image was saved in TIFF format, a lossless format. Figure 6a shows the scanned image of the paper microtiter plate with the conducted test for GSH detection. The extracted RGB mean values (n = 3) with error ranges were plotted versus the GSH concentration in Fig. 6b. It was found that the value of RGB mean increased with the increase in GSH concentration. But, the linearity decreased beyond 90 µM as the values of RGB mean were deviating from the trend. Therefore, for further studies, 0–90 µM was considered as the concentration range.

The relationship between the ratios of RGB values (i.e., (log (RGBf/RGBi))) against the different GSH concentrations has been plotted to obtain the calibration plot, where RGBf and RGBi are the RGB mean values in considered and initial conditions, respectively (Nghia et al. 2020; Monisha et al. 2021). The result showed an excellent linear correlation ($R^2 = 0.989$), which has been depicted in Fig. 7. The LOD was calculated using the equation $\text{LOD} = 3 \times (\sigma/m)$ where, $\sigma$ and $m$ were the standard deviation and slope of the linear fit, respectively (Boruah et al. 2019). The curve showed a LOD of 28.375 µM. The feasibility of conducting such colorimetric tests on the fabricated paper microtiter plate emphasizes its potency for usage in various bioanalytical tests in real environments.

To further assess the competency of the fabrication strategy, recent reports have been compared centralizing the fabrication technique utilized to realize the paper-based analytical platforms, and have been tabulated in Table 1. It can be observed that the compared techniques utilized specialized equipment whereas the proposed methodology challenges such requirements and allows a facile and rapid realization alternative. The stability of the fabricated microtiter paper plate has been evaluated by keeping it at 40 °C in an oven for 50 h. The characterization procedures showed no change in the shape and size of the wells. Thus, the wax patterns did not melt at elevated temperatures and worked as a microtiter paper plate. Moreover, the validated colorimetric detection methodology of GSH conducted on the fabricated plate with reasonably good analytical performance further instigates the functionality.

![Fig. 7 The linear calibration plot of log (RGBf/RGBi) versus GSH concentrations from 0 to 90 µM](image)

| S. No. | Platform                  | Fabrication technique       | Specific requirements                   | Detection strategy      | Analyte                        | References                      |
|-------|---------------------------|------------------------------|-----------------------------------------|-------------------------|-------------------------------|--------------------------------|
| 1     | 96-well plate on paper    | Thermal printing                      | Thermal transfer portable printer (HPRT MT800 2.0) | Colorimetric detection | HRP                           | Ruiz et al. (2022)              |
| 2     | µPAD                      | Inkjet printing                | Inkjet printer (HP deskjet printer 1112) | Colorimetric detection | Candida albicans (pathogenic fungi) LOD 0.86 × 10^6 CFU/ml | Prabhu et al. (2020)            |
| 3     | µPAD                      | Direct writing                 | 3D printer                              | Colorimetric detection | Aflatoxin B1                  | Mirón-Mérida et al. (2020)      |
| 4     | 96-well plate on paper    | Wax printing                   | Wax printer (Color Qube 8580 n)         | Colorimetric detection | Saccharides                  | Lyu et al. (2021)               |
| 5     | LP-µPAD                   | Solid ink toner printing        | Laser printer (HP LaserJet Pro P1606)   | Colorimetric detection | Nitrite and E. coli LOD 250 µM | Ghosh et al. (2019)             |
| 6     | µPAD                      | PDMS stamping                  | Laser engraving machine                 | Colorimetric detection | Aflatoxin B1 LOD 9.54 ng/ml   | Tang et al. (2022)              |
| 7     | 96-well plate on paper    | Wax stamping                   | No specific equipment                    | Colorimetric detection | Glutathione LOD 28.375 µM     | This work                      |
of the fabrication technique. The reported linear detection range is comparable with the colorimetric detection strategy of Chen et al. (2021) involving carbon nanoparticles as peroxidase-mimics. They reported a detection range of 2.5–50 µM with a limit of detection of 0.26 µM. Similarly, Sun et al. (2022) synthesized core–shell Cu/Au nanoparticles possessing peroxidase mimicking characteristics for detecting GSH and reported a detection range from 0.07 to 1.43 mM with a LOD of 13 µM. However, it is worth mentioning that the current work does not employ any synthesized nanomaterials or uses any sophisticated processes to detect analytes with improved analytical performance. Nonetheless, a congenial study involving paper microtiter plates reported a LOD of 250 µM for detecting saccharides (Lyu et al. 2021). Henceforth, the proposed fabrication methodology holds a strong candidature for an equipment-free realization of a 96-well microtiter paper plate.

4 Conclusion

In this work, a novel technique to fabricate a 96-well microtiter plate on paper has been presented. The technique implicated the usage of polystyrene microtiter plates as a stamp to pattern wax on the paper substrate. The strategy allowed rapid prototyping of 96-well paper microtiter plates. The process also indorsed the recycling of used polystyrene plates for fabricating paper microtiter plates and thus envisaged reduced generation of non-biodegradable waste. Apart from this, the advantage of facile fabrication, minimal requirements of reagents, and shorter processing time of PADs in general, further advocates its utility in various diagnostic applications. The practicality of the fabricated platform has been assessed by executing colorimetric tests for GSH detection. The results depicting a linear range in GSH concentrations from 0 to 90 µM with a LOD of 28.375 µM using RGB analysis complements the practicality of the fabrication technique to realize a 96-well microtiter plate on paper substrates.

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Author's contribution MB: designing, execution of experiments and manuscript drafting. DM: designing, execution of experiments and manuscript drafting. HSD: Conceptualization and designing of experiments, manuscript drafting and reviewing.

Declarations

Conflict of interest The authors declare that they have no known competing interests that could have appeared to influence the work reported in this paper.

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