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A novel direct and cost effective method for fabricating paper-based microfluidic device by commercial eye pencil and its application for determining simultaneous calcium and magnesium

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ABSTRACT

This article presents a novel, simple and low-cost method for the fabrication of microfluidic paper using sticker templates with specific patterns and a highly accessible waterproof eye pencil. The entire fabrication process could be finished in 10 min by heating the patterned paper at 150° without using complicated instruments. Important parameters in the fabrication of microfluidic paper such as type of paper, heating time, melting temperature, reagent and sample volume were optimized. To verify the applicability of the fabricated microfluidic paper, colorimetric assays were performed for simultaneous analysis of calcium and magnesium by single and multiple indicators and water hardness was determined. The limit of detection is 8.3 mg L^{-1} and 1.0 mg L^{-1}, and the relative standard deviation is 8.3% and 5.9% for calcium and magnesium, respectively. A linear range from 10 to 100 mg L^{-1} for calcium and two linear ranges of 4–20 mg L^{-1} and 20–100 mg L^{-1} for magnesium were obtained in the paper based microfluidic device. The concentration of calcium and magnesium were successfully determined in tap, river, mineral and household purifier water samples.

1. Introduction

Paper-based microfluidics devices (μPADs), introduced by Martinez et al. in 2007, have multiple advantages such as low cost, low sample volume, portability, disposability, non-instrumented and multi-analyte analysis [1–3]. Furthermore, they allow quantitative measurement using a μPAD based on a variety of detection methods. Colorimetric assays on paper are widely used to quantify the color intensity of the test zone due to the ease of use which 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 [8–10], transmittance [11,12], fluorescence [13,14], chemiluminescence (CL) [15] and electrochemiluninescence (ECL) measurements [16]. Currently, μPADs has been the subject of growing attention and numerous methods have been proposed for their fabrication. Including photolithography [1,17,18], wax printing [19], inkjet printing [20], screen-printing [21], direct writing of polydimethylsiloxane (PDMS) [22], paper cutting [23] and so forth. One limitation of the above fabrication methods is that they need tools that rarely found in laboratories of developing countries. Moreover, the usage and maintenance of these tools require personnel.

This paper proposes a novel method for the fabrication of paper-based microfluidic devices by a commercial eye pencil. These pencils are easily accessible, cheap, easy-to-use and non-toxic. This fabrication method requires only a hot plate for the patterning of hydrophobic and hydrophilic areas on the Whatman paper, which can be performed by a simple dry iron as well. The fabrication of the μPAD is simple, fast and solvent free. To demonstrate its applicability to real world situations, the paper device was also used for simultaneous colorimetric assays of calcium and magnesium in water samples. The general methods used for determining calcium and magnesium are complexometric titration [24], spectrophotometry and atomic absorption spectrometry. The first one, complexometric titration, is the classical method used for determination of calcium and magnesium in water samples where it is impossible to obtain the amount of Ca^{2+} and Mg^{2+} directly. To do so, we first need to determine the total amount of calcium and magnesium, followed by a determination of calcium, and then magnesium based on the difference of the first two steps, which makes this method relatively complex [25]. In the spectrophotometric method, the simultaneous determination of calcium and magnesium is hampered due to spectral overlap of their complexes [26–29]. In this regard, atomic absorption spectrometric methods are more sensitive, but they also more expensive [30–32].

This paper presents the paper platform uses for simultaneous colorimetric determination of calcium and magnesium in samples without any pretreatment in short times that according to the author's

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knowledge, has not reported in the literature. The results of assays were satisfactory and significantly similar to those of titration methods.

2. Experiment

2.1. Reagents and equipment

Calcium chloride dehydrate, Magnesium nitrate hexahydrate and Ethylene glycol-bis (2 aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA) were purchased from Sigma Company (Sigma-Aldrich Química S.A., Spain). Xylidyl blue, Eriochrome black T (1 - (1-hydroxy -2 -naphthylaxo)-2 -hydroxy - 5-nitro - 4 -naphthalenesulfonic acid), Ethanolamine and Murexide (ammonium purpurate) were also acquired from Merck (Merck Millipore, Germany). All reagents were of analytical purity grade and prepared on a daily basis.

Whatman filter papers No. 41 (215 μm thick, 20–25 μm pore size), No. 42 (200 μm thick, 2.5 μm pore size) and No. 40 (210 μm thick, 8 μm pore size) were purchased from Whatman International, Ltd. (Maidstone, England) and the water proof eye pencil was purchased from Bourjois Paris. A D-500 Alfa hot plate (Tehran, Iran) was used for fabrication of **μ**PADs and a CanoScan LiDE 120 Color Image scanner was employed for colorimetric detection. A micropipette (Eppendorf Research® 0.1–2.5 μL) was used for injection of reagents and samples.

2.2. Fabrication and preparation of the paper-based microfluidic devices

We used graphics software (Auto cad 2014) to design a μPAD. The details of the design are shown in Fig. 1. The design was cut by a CO_2 laser on white sticker papers and a cut sticker was put on Whatman® No. 41 filter papers, followed by the application of the eye pencil to draw around the cut stickers placed on the filter paper. Then, the eye pencil was spread with a tissue paper on the filter paper. Finally, the filter paper was placed on a digital hot plate (150 °C, 5 min), which allowed the eye pencil to diffuse vertically through the porous paper and created hydrophobic barriers that defined hydrophilic channels. Finally, the sticker was separated from filter paper. One side of the paper substrate was then covered by a packing tape to prevent the leakage of eluent through the bottom of the PAD. A video showing the experimental procedures is also attached in the Supporting Information.

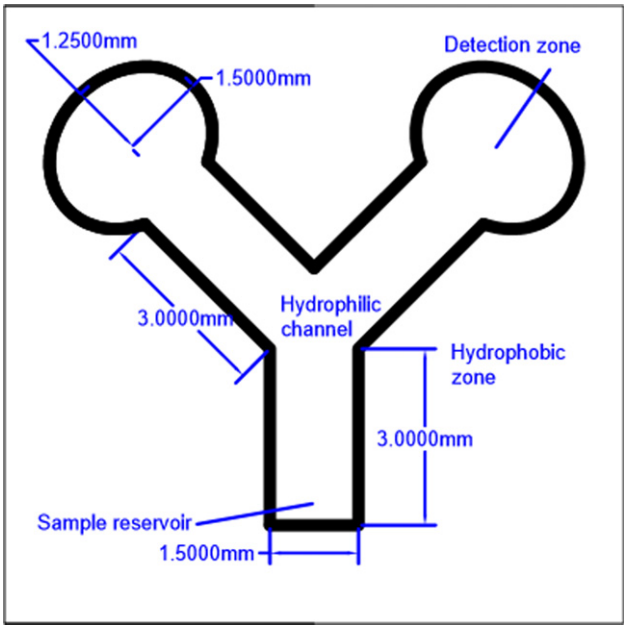

Fig. 1. The design of μPAD.

2.3. Colorimetric detection

2.3.1. Spotting of reagents on the detection zone

For Mg assay, a solution of 1 mol L^{-1} Ethanolamine, 110 μmol L^{-1} Xylidyl blue and 160 μmol L^{-1} GEDTA was prepared (solution I). Then, a 2% Eriochrome black T solution was prepared by dissolving 0.05 g of the reagent in 2.5 mL Ethanol and applying a filter paper (solution II). The Mg detection reagent consisted of two solutions that were spotted on top of each other in the detection zone of the paper device. Initially, 0.1 μL of solution I (four times) was spotted in the detection zone of the paper device, which was allowed to dry for 5 min. Then, 0.1 μL of solution II was spotted directly on top of the spot of solution I and was left to dry for 5 min.

For *Ca* assay, a 2% solution of murexide was prepared by dissolving 0.05 g of reagent in 2.5 ml water, and it was filtered to prevent the formation of a nonhomogeneous color. In Fig. S1, a comparison of detection zone for assay Ca before and after filtration is shown. The μPAD was prepared by adding 0.1 μL aliquots of murexide to the detection zone by the micropipette.

2.3.2. Addition of standard solution

A stock solution of calcium and magnesium (200 mg L^{-1}) was prepared by dissolving 0.0735 g of calcium chloride and 0.2136 g of magnesium nitrate hexahydrate in 20 mL water, which was then diluted to 100 mL with deionized water. The working standard solution for the calcium and magnesium was made by appropriate dilution of the stock solutionon a daily basis as required.

The mixture of Mg and Ca standard solution was prepared in the range of 1–120 mg L^{-1} for Ca and Mg. 1.4 μL of the analytical sample was pipetted to the μPAD and transferred through the hydrophilic channels to the detection zones. Both indicators changed color simultaneously as the mixture of Mg and Ca standard solution reached the detection zones (see supplementary data). The μPAD was finally maintained at room temperature for additional 15 min to allow the stability of color development in the detection zone.

2.4. Image processing

The color was taken by flatbed scanner (600-dpi resolution) and analyzed using Adobe Photoshop CS5. In all cases, a detection zone with appropriate tolerance level was selected to analyze the image in CMYK color space using the yellow channel for Ca and magenta channel for Mg. To obtain background corrected data, the mean pixel intensity of each μPAD was measured before the deposition of analyte (as the blank measurement). Again, after pipetting the sample solution containing calcium and magnesium, the mean pixel intensity of each device was measured (as the analyte measurement). Finally the absorbance was calculated as the negative 10 base logarithm of the ratio of color intensity of test zone containing analyte and the blank (Fig. S2).

3. Results and discussions

3.1. Optimization of the μPAD fabrication

The following parameters were optimized:

3.1.1. Effect of the paper type

There are a variety of filter papers available to the user, though the selection criteria are based on the fabrication steps required for developing a device and the specific application area [33]. In this work, we examined the effect of three types of Whatman® filter papers (No. 41, No. 40 and No. 42) with different pore sizes. For example, Fig. 2, the difference between Whatman® filter paper No. 41, 40 and 42 after hydrophobization is shown. Paper No. 41 which has the largest pore size and thus the highest flow rate among all three filter papers, was appropriate because after exposure to heating at 150 °C for 5 min (Fig. 2,

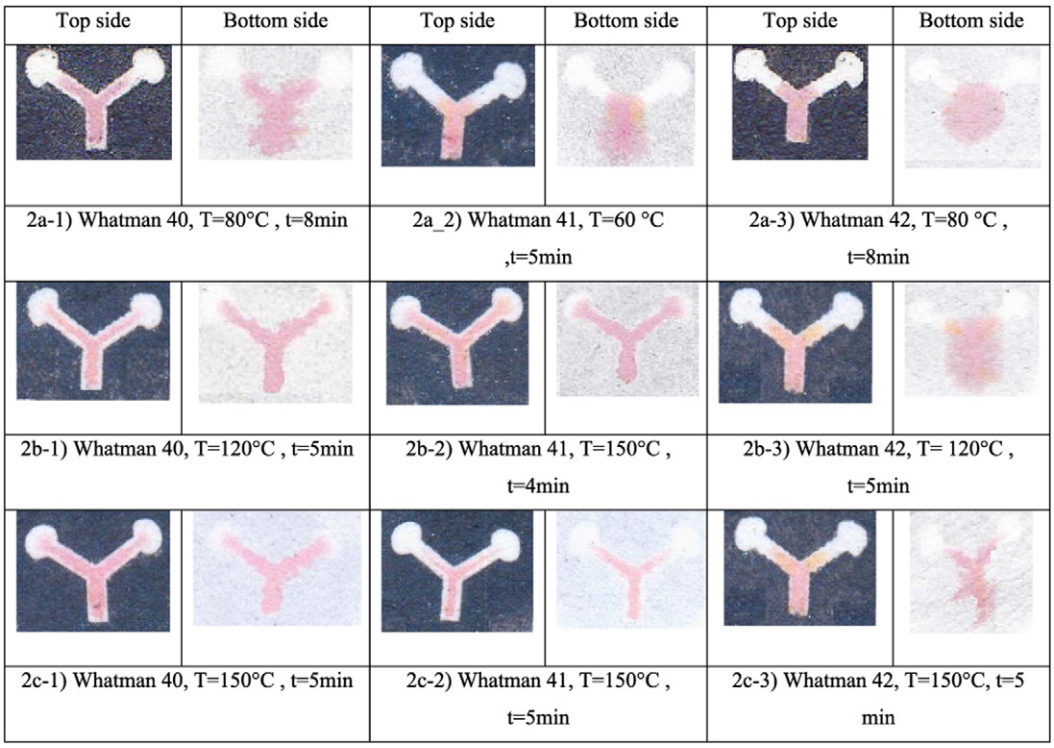

Fig. 2. Molten eye pencil spreading as a function of temperature, time, and type of filter paper.

2c-2), the molten eye pencil could easily penetrate the paper and hydrophobization was achieved more completely.

3.1.2. Effect of heating time and melting temperature

To verify best conditions for the fabrication of microfluidic paper-based device, a set of experiments –were performed to optimize effective parameters -in terms of time and temperature needed to melt the eye pencil on the paper with the aim of producing impermeable barriers across the entire thickness. As shown in Fig. 2, at a temperature below 150 °C and time intervals <5 min, the hydrophobic and hydrophilic areas were not separated exactly. On the other hand, the application of a melting temperature above 150 °C and heating time longer than 5 min resulted in the spread of excessive molten eye pencil into the paper and also can burn the paper. The best spreading ratio achieved at full penetration was 5 min at 150 °C using Whatman® filter paper No. 41.

3.1.3. Effect of reagent and sample volume

We determined the reagent volume required to wet the entire detection zone by dropping murexide indicator solution. As shown in Fig. S3, 0.15 μL indicator solution spread outside the detection zones and dispense <0.1 μL is impossible with standard micropipettes. Furthermore, any volume <0.1 μL of reagent solution would be unable to completely wet the detection zones. Therefore, 0.1 μL of reagent solution was selected for further experiments.

The minimum sample volume that could spread through the entire device was also studied by spotting murexide indicator solution into

				Mean color intensity in Magenta channel
Indicator	**Xylidyl blue**	Before spotting Magnesium		227
		After spotting Magnesium		223
	Eriochrome black T	Before spotting Magnesium		176
		After spotting Magnesium		173
	Mix Eriochrome black T & Xylidyl blue	Before spotting Magnesium		184
		After spotting Magnesium		120

Fig. 3. Color intensity and color discrimination difference between a single indicator and a mixture of two indicators for colorimetric detection.

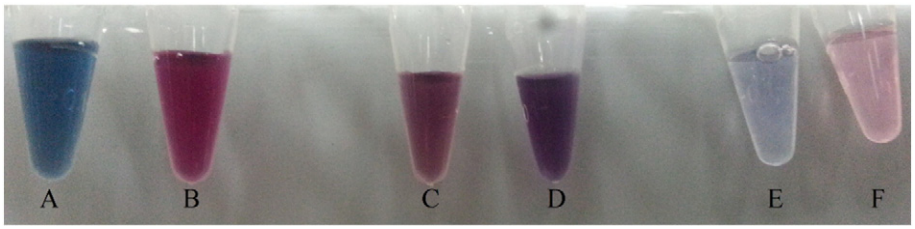

Fig. 4. Comparison of a single indicator and a mixture of two indicators. A) Mix Eriochrome black T & Xylidyl blue without magnesium, B) Mix Eriochrome black T & Xylidyl blue with Mg, C) Eriochrome black T without magnesium, D) Eriochrome black T with magnesium, E) Xylidyl blue without magnesium, F) Xylidyl blue with magnesium.

the sample reservoir. It was found that 1.4 μL of sample was required to fill all detection zones (Fig. S4).

3.2. Colorimetric assay

In this work, two indicators including Eriochrome black T and Xylidyl blue were evaluated to determine Mg. Magnesium forms a colored complex with these indicators. In an alkaline medium, Eriochrome black T is blue in color but after reaction with magnesium, it produces a red-violet color. Also, the reaction of magnesium ions with Xylidyl blue produces a red complex. A greater visual difference is possible when more than one color is developed as opposed to discriminate hues or intensities of a single color. Hence, multiple indicators should provide more accurate results as compared to single color tests by allowing differences in hue and intensity to be averaged across multiple detection spots for the same analyte [2]. In Fig. 3, the comparison of a single indicator and a mixture of two indicators is shown.

The results are shown for the same proportion in Fig. 4.

Calcium interference is virtually eliminated by the use of EGTA [34]. Magnesium can be spectrophotometricly determined at 546 nm, but the only shortcoming of this method is the colored chelate is only stable for 20 min, after that the colored solution is turbided and precipitated so that the absorbance determination is impossible. As a result, it must be read immediately.

For determination of Ca, murexide was used. In a relatively neutral solution, the reaction of murexide with calcium ions leads to a color change from pink to reddish yellow [35,36].

3.3. Analytical performance

3.3.1. Analytical figure of merit

In Fig. S5 and, both the qualitative and quantitative calibrations of Mg and Ca are shown under optimal conditions for the proposed paper-based method. The calibration plot used for determining magnesium is linear in two concentration ranges of 4–20 mg L^{-1} and 20–100 mg L^{-1}. A detection limit of 1.0 mg L^{-1} ($n = 6$) was obtained. The detection limit is defined as a concentration equivalent to 3-folds of standard deviation of log color intensities for 6 blank samples (the blank sample was prepared by spotting deionized water to μPAD). Also, the estimated pooled relative standard deviation (RSD_{pooled}) [37] for all measurements ($n = 6$ for each Mg level) was 5.9%. The RSD showed an excellent reproducibility of the proposed method. The calibration equations are Absorbance (Mg) $= 4.2 \times 10^{-3} C_{Mg}$ (mg L^{-1}) $+ 1.6 \times 10^{-2}$ ($R^2 = 0.9971$) for the range of 4–20 mg L^{-1} and Absorbance (Mg) $= 9.0 \times 10^{-4} C_{Mg}$ (mg L^{-1}) $+ 8.0 \times 10^{-2}$ ($R^2 = 0.9971$) for the range of 20–100 mg L^{-1}.

Also, the proposed paper-based method was characterized by linear calibration ranges for calcium 10–100 mg L^{-1}, with calibration equations of Absorbance (Ca) $= 1.7 \times 10^{-3} C_{Ca}$ (mg L^{-1}) $+ 1.0 \times 10^{-2}$ ($R^2 = 0.9951$). The limits of detection (LOD) for calcium was 8.3 mg L^{-1} ($n = 6$) and a RSD_{pooled} of 7.6% was achieved for all measurements ($n = 6$ for each Mg level).

3.4. Interferences study

The interference of common ions in water such as K^+, Na^+, Cl^-, PO_4^{-3}, SO_4^{-2}, Fe^{+3}, NO_3^-, I^-, F^-, Al^{+3}, Ni^{+2} and Mn^{+2} was studied. Among them, only the presence of Ni^{+2} and Mn^{+2} in high concentrations interference the determination calcium and magnesium. Ni^{+2} and Mn^{+2} within 1 mgL^{-1}, K^+, Na^+, Cl^-, PO_4^{-3}, SO_4^{-2}, Fe^{+3}, NO_3^-, I^-, F^- and Al^{+3} do not affect the results.

3.5. Real samples

To determine the hardness of water, the proposed μPAD was used to determine calcium and magnesium in different water samples including tap, river, mineral and household purifier water. To confirm the validity of the present method, the assay results were compared to those of the titration method [24]. The water samples were directly analyzed by μPAD and titration. The results of analysis are shown in Table 1. A comparison of results showed that both methods were significantly similar so that the paired *t*-test analysis did not show any significant difference between the two methods at a 95% confidence level [38]. Therefore, this device could be used to simultaneously determine calcium and magnesium concentrations in water samples.

3.6. Comparison with other methods

Some analytical data reported for determination of water hardness are compared with the proposed method in Table 2. Some of these methods, such as UV–vis, Flame atomic absorption spectroscopy require expensive equipment. Furthermore, in the UV–vis method, the spectra of calcium and magnesium were completely overlapping. However multivariate analysis must be used and requires certain skills like chemometrics techniques. In complexometric titration by EDTA, though inexpensive, is time consuming and subject to operational errors. Also,

Table 1
Comparison of calcium and magnesium concentration levels in water samples obtained by titration and colorimetric measurements with fabricated μPADs ($n = 5$).

Samples	Calcium (mg L^{-1})		Magnesium (mg L^{-1})		Total hardness	
	This method	Titration	This method	Titration	This method	Titration
Tap water (Ferdowsi University of Mashhad, Iran)	60.7 ± 6.6*	57.1 ± 2.3	18.1 ± 2.4	18.9 ± 1.4	78.8 ± 7.0	76.0 ± 2.7
River water (Hesar river, Torbat-e Heydarieh, Iran)	68.4 ± 5.3	64.5 ± 2.3	20.5 ± 0.4	19.8 ± 1.4	88.9 ± 5.4	84.3 ± 2.7
Mineral water (Nestle, Iran)	48.2 ± 4.2	44.3 ± 2.3	7.8 ± 2.2	7.4 ± 1.4	56.0 ± 4.7	51.7 ± 2.7
Household purifier water (Puritec, Taiwan)	30.4 ± 3.7	28.3 ± 2.3	6.8 ± 1.8	7.0 ± 1.4	37.2 ± 4.1	35.3 ± 2.7

*Confidence limit

Table 2
An overview on recently reported optical methods for determination of water hardness (Mg^{2+}/Ca^{2+}).

Analyte	Analytical technique	Linear range	References
Ca^{+2} & Mg^{+2}	Classic complexometric titration	2–100 mg L^{-1}	[39–41]
Ca^{+2} & Mg^{+2}	Flame atomic absorption spectroscopy (FAAS)	3–50 mg Ca L^{-1} and 0.9–5 mg Mg L^{-1}	[39,42]
Total hardness	UV–vis	1.9–14,800 mg L^{-1} as $CaCO_3$	[43]
Ca^{+2} & Mg^{+2}	UV–vis	0.1–4 mg Ca L^{-1} and 0.15–2.5 mg Mg L^{-1}	[27]
Ca^{+2}	Camera	0.2–2.0 mg L^{-1}	[44]
Ca^{+2} & Mg^{+2}	Scanner	Two linear ranges 4–20 mgL^{-1} & 20–100 mg L^{-1} for Mg and 10–100 mg L^{-1} for Ca	Proposed method

all the methods listed in Table 2 have a linear range in the mgL^{-1} range and in this respect, not differences. Furthermore, there are commercially available paper strips that they measure the total hardness of water and this paper strip cannot determine the concentrations of Ca^{+2} and Mg^{+2} independently. And also this paper strip reports that the hardness of the sample is <55, >90, >180, >270, >360, or >445 mgL^{-1}. Conversely, the proposed μPAD permit the determination of
the exact concentrations for both Ca^{+2} and Mg^{+2}.

4. Conclusion

In this paper, a novel method for the fabrication of hydrophobic/hydrophilic structure by a commerical eye pencil and patterned stickers was proposed. Fabrication procedure was performed by heating the patterned paper at 150° by a hot plate for about 10 min. This method was simple, quick and inexpensive without need for complicated and expensive instruments. Furthermore, the proposed paper based microfluidic device was used for colorimetric determination of calcium and magnesium with a flatbed scanner in different water samples. The results of μPAD were compared to those of titration method and good agreement was observed. The proposed fabrication method can be used in developing countries with limited resources.

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Appendix A. Supplementary data

Two videos of the procedure of the fabrication of the paper-based microfluidic devices and running of the experiments are available in the supporting information. Fig. S1 showed a comparison of detection zone for assay Ca before and after filtration. Fig. S2 showed the image processing for analyzing calcium and magnesium using Adobe Photoshop software. Fig. S3 and Fig. S4 showed the optimum reagent and sample volume, respectively. Fig. S5 and S6 showed the qualitative and quantitative calibrations of calcium and magnesium. Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.microc.2017.04.031.

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