A Portable Paper Based Device for Quantitatively Colorimetric Assays Relying on Light Reflectance Principle.

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Research Article

Portable paper-based device for quantitative colorimetric assays relying on light reflectance principle

This paper presents a novel paper-based analytical device based on the colorimetric paper assays through its light reflectance. The device is portable, low cost (<20 dollars), and lightweight (only 176 g) that is available to assess the cost-effectiveness and appropriateness of the original health care or on-site detection information. Based on the light reflectance principle, the signal can be obtained directly, stably and user-friendly in our device. We demonstrated the utility and broad applicability of this technique with measurements of different biological and pollution target samples (BSA, glucose, Fe, and nitrite). Moreover, the real samples of Fe (II) and nitrite in the local tap water were successfully analyzed, and compared with the standard UV absorption method, the quantitative results showed good performance, reproducibility, and reliability. This device could provide quantitative information very conveniently and show great potential to broad fields of resource-limited analysis, medical diagnostics, and on-site environmental detection.

Keywords:
Colorimetric assay / Light reflectance / Metal ions / Nitrite / Paper-based microfluidic device / Point-of-care
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Additional supporting information may be found in the online version of this article at the publisher's web-site

1 Introduction

Currently, health care workers are lack of adequate equipment to carry out the diagnosis of common diseases in resource-limited settings, and therefore the development of low-cost diagnostic technique, portable and truly affordable devices in these countries have become extremely necessary [1, 2]. These instruments cannot be bulky, expensive, and do not require strong expertise available to make the original unattainable health care. Meanwhile, environmental pollution has become increasingly a serious problem. The traditional monitoring methods are also facing the similar problem such as samples storage and transportation, the high cost of monitoring, higher skills, and rich experience required. Thus, a simple, robust, and portable environmental monitoring technology is highly desired.

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Abbreviations: HRP, horseradish peroxidase; **NEDD**, *N*-(1-naphthyl)-ethylenediamine dihydrochloride; **μPAD**, microfluidic paper-based analytical device

Microfluidics or lab-on-chip as a promising and powerful platform shows great potential in the past two decades [3–8]. And recent paper microfluidics technology developed very rapidly depending on those attractive features such as cheapness, simple operation, lightweight to transport, and easy-to-use. That let it be easily accessible to point-of-care diagnostic and promote the development for the issues of on-site detection [9] and environmental monitoring. Whitesides et al. provided the concept of microfluidic paper-based analytical devices (μPADs) at 2007 [10]. They attempted to pattern hydrophobic SU8 gel by photolithography on the paper and its hydrophilic structure that could work for the transportation of liquid through capillary force, not requiring the external equipment. Besides photolithography manner, various printing methods have been tried out and established, including inkjet printing [11, 12], wax printing [13, 14], and screen printing [15], etc. Currently, wax printing becomes a feasible method depending on its characteristics of fastness and convenience. Meanwhile, other fabrication methods such as paper cutting [16], spray [17], stamping [18], origami [19,20], and laser treatment [21] also have been developed. With the development of μPADs, this technology has been quickly applied to a number of low-cost and user-operated devices such as colorimetric detection [22–26], electrochemical detection [15,27,28],

Colour Online: See the article online to view Figs. 1–4 in colour.

chemiluminescence biosensor [29–31], MS [32, 33], surface-enhanced Raman spectroscopy (SERS) [34, 35], cell culture [36, 37], and blood analysis [38, 39], etc.

In conventional test strip, the answer with "yes/no" or semiquantitative result was not enough. People need more accurate quantitative information for the on-site detection or the personal disease diagnosis. Currently, most people used mobile phone with camera as the detection devices and the software would calculate the values by converting images of paper assays from color scale into grayscale [40, 41]. However, they sometimes suffered from the variable changes of light condition. Herein, some people used scanner to implement testing. But it was difficult to be portable for on-site detection. However, these methods had some disadvantages. One was the specific software such as Photoshop using for calculation that were expensive for individuals. Moreover, the data converting process was tedious and required good expertise. Another one was that the variable environment light's interference cannot be avoided. In order to solve these problems, Fang et al. obtained quantitative assay results using the reflected optical fiber [42]. However, the fiber optical testing system was less of integration. Ellerbee et al. demonstrated a novel colorimetric device by measuring the transmission of light through paper at 2009 [43]. It was an affordable and hand-held device that was very suitable to the diagnosis or resource-limited detection. However, it had some limitations. For instance, a lot of light energy lost when the light passed through paper, which resulted in the serious decrease of signal. Moreover, the process was tedious because it needed to wet the paper with vegetable indexing oil [44, 45], and the different thickness of paper greatly affected the accuracy of the measurements.

In order to address these issues, we firstly provide the new design of paper-based device relying on light reflectance principle. Though there are some glucose meters commercial, they only can monitor the glucose level in the blood, but not for all colorimetric assays. Herein, our device could detect many colorimetric assays quantitatively including glucose and nonglucose targets. And as a proof-of-principle demonstration, some biological, and pollution target samples (BSA, glucose, Fe, and nitrite) had been successfully analyzed. This device was portable, lightweight (Supporting Information Fig. 1), inexpensive, and could provide reproducible results. Due to the fully closed testing environment and the steady LED light source, the detection signal was stable and hardly prone to environment light's interference. Even in dusty or bad weather, it could also work properly and this advantage made it ideal for outside and on-site detection. Compared with smart phone, the complicated and tedious data converting process was no longer needed and the signal value could be easily read-out from the LCD (liquid crystal display) screen. The total cost of the equipment was \$18.2 (details listed in the Supporting Information Table 1). With the attractive features such as portability, rapidity and low-cost that would have potential to be used in the field of the diagnosis, resource-limited, or on-site detection.

2 Materials and methods

2.1 Materials

Glucose, BSA, glucose oxidase enzyme, and horseradish peroxidase (HRP) were obtained from Sigma-Aldrich. Tetrabromophenol blue (TBPB) were purchased from J&K Scientific company. HCl, H_3PO_4, NaOH, KI, $NaNO_2$, PVA, *N*-(1-naphthyl)-ethylenediamine dihydrochloride (NEDD), *p*-aminobenzene sulfonic acid (PABS), hydroxylamine, citric acid, sulfonamides, and 1,10-phenanthroline (Phen) were purchased from Sinopharm Chemical Reagent (China). All chemicals were analytical reagent grade or better and all aqueous solutions were prepared with doubly distilled water (18.2 MΩ, Pall Cascada laboratory water system). The safety precautions were carried out by handling of chemicals with their respective Material Safety Data Sheet.

The baking equipment was an oven from Boxun industry & commerce company (Shanghai, China). The Whatman filter paper (No#1) was from Hangzhou Xinhua Paper Limited Company (GE Healthcare, China). The 8560DN wax printer was purchased from Fuji XEROX Company. The 3.7 V cellphone battery was purchased from Pisen Company (China).

2.2 Fabrication of portable colorimetric devices and paper strip

The schematic layout of the portable device was demonstrated in the Fig. 1. The device was composed of ARM (Advanced RISC Machines) stm32, newly color sensor chip, three LED lights, battery, LCD display screen, and other components. The light-to-digital converter color sensor chip was purchased from TAOS company that was a latest generation product and had good reproducibility and sensitivity with small dimension. The ARM stm32 was used to control all the parts including data collection, calculation, and the display on the screen automatically written by C language. The color-sensing chip was used to distinguish and digitize color change. A 3.7 V cell phone battery was used to provide long-time power. The Nokia LCD screen was selected to achieve the results of colorimetric assays. In order to allow the brightness of light more uniformly for chip detection area, three LED lights matrices were distributed around the color sensor chip as the center with the angle of 120 degrees (Fig. 1E). When the detection area was excited by three LED lights, the reflection signal was collected by the color sensor chip and its resolution could be up to 16 bits for RGB color sensing. The sample-collecting rate was 100 millisecond and ten adjacent data to conduct a smooth data after ARM processing. Then the smooth data containing the quantitative information from colorimetric assays could be readout immediately. To be free from outside interference, the testing environment was fully closed by inserting the sleeve to the portable device.

The manifold and the paper's dimension were illustrated in Fig. 1A and B. The fabrication process was performed

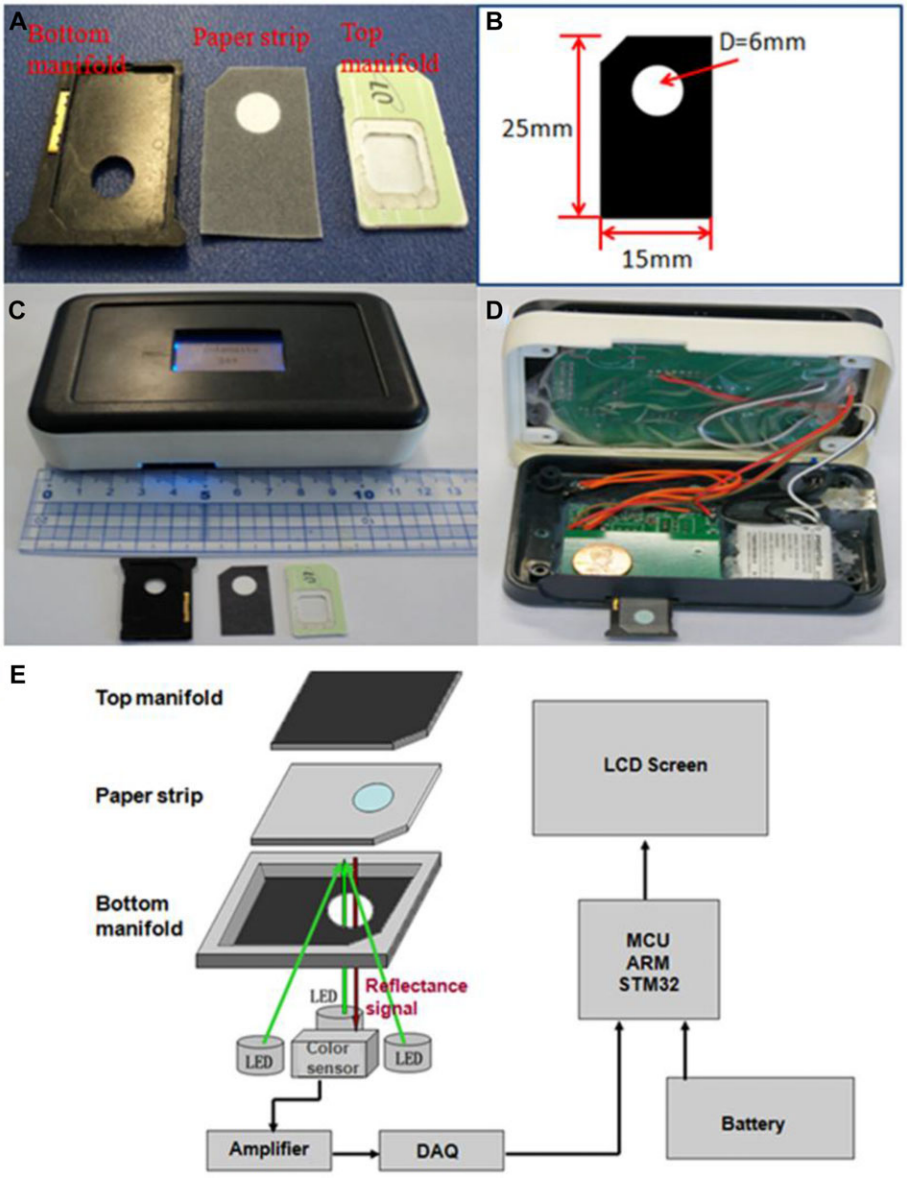

Figure 1. (A) The bottom (left), the top (right) plastic manifold, and the paper strip (middle) can be placed well in this manifold. (B) The dimension of the paper strip is 15 mm × 25 mm and the detection area's diameter is 6 mm. (C) The hand-held attachment has dimensions of 11.8 × 7.2 × 2.0 cm^3. (D) The internal layout of the device. The paper chip needs to be inserted into the sleeve and the detection area is near the right of the coin. The whole detecting environment is fully closed. (E) Schematic layout of the paper-microfluidic portable device to measure the reflectance light. The color sensor chip is placed in the downward vertically direction of the manifold and the three LED lights were distributed around the color sensor chip as an angle of 120 degree. The reflectance signal were collected, amplified and processed by MCU (micro control unit) ARM and the smooth data were shown on the screen.

following by previously described methods [14, 46]. The paper strip's size was 15 × 25 mm that fitted well for the manifold and the round-shape hydrophilic region was in the middle with 6 mm diameter. The top manifold could completely avoid light and there was a circular hole (6 mm diameter) at the bottom manifold for detection. When measuring the reflection of light from paper, the paper strip was put to the sleeve (Fig. 1C and D) and the color sensor was placed vertically downward direction of the strip to collect the signal.

The chip design was implemented by drawing software (Adobe Illustrator) and then directly printed onto the filter paper (Whatman chromatography No. 1 paper, GE) by a wax printer (XEROX Phaser 8560DN) with highest resolution 2400 dpi. After printing, the paper strip was kept in an oven at 150°C for 2 min to let the wax to penetrate through the paper completely. In this way, the 3D hydrophobic wax barriers could prevent the liquid diffusion to the outside. And its diameter was equal to the diameter of paper's detection area as same as 6 mm (after wax melting). There was a plastic manifold at the top to fix the paper strip in proper position.

Furthermore, we provided a whole tutorial via a movie (see Supporting Information) including paper's detection process and signal's readout. The blank paper (white color) and a green color paper were used as testing examples in the movie. The paper strip could be easily assembled into the device and the results were displayed in the LCD screen with different intensities for different colorimetric assays.

2.3 Samples preparation

2.3.1 Glucose assay

The potassium iodide (KI) and HRP were 0.6 M and 5×10^{-5} M, respectively. Sodium acetate (NaAc) concentration was 50 mM (pH 5.1). The 0.45 mg glucose oxidase enzyme was solved in 1 mL 50 mM NaAc buffer. The 0.18 g glucose was solved in 10 mL H_2O with the concentration as 100 mM. Three microliters KI and 3 μL mixture of HRP and glucose oxidase enzyme were added respectively after the paper dried. Finally, the 3 μL different concentrations of glucose were added by micropipette, and the reaction time was 25 min.

2.3.2 BSA assay

The 3 μL 250 μM citrate buffer (pH 1.8) was firstly added on the paper. Then 3 μL 3 mM TBPB (solved in the 95% ethanol) was added. Both solutions were air-dried 10 min at room temperature. The 0.034 g BSA was solved in 5 mL H_2O and the final concentration was 100 μM, and then diluted to lower concentrations for use. Finally, the 3 μL different concentrations of BSA were added by micropipette, and after 2 min reaction, the assays were detected by the portable device.

2.3.3 Nitrite assay

Nitrite firstly had a diazotization reaction with the sulfanilic acid and then makes coupling with NEDD to produce purple azo. The mixture of 50 mL H_3PO_4 ($\rho = 1.70$), 20 g PABS, and 1 g NEDD were solved in DI water with the final volume as 500 mL for chromogenic reagent. The nitrite was prepared as different concentrations (50, 25, 15, 10, 5, 3, 2, 1 mg/L). Three microliters NEDD chromogenic reagent was added to the detection area and air-dried in 10 min at room temperature. Then 3 μL different concentrations of nitrite were introduced to the detection areas and kept a color reaction 2 min for determination.

2.3.4 Fe assay

Ferrous ions have a reaction with phenanthroline in the pH value of 3–9, and generates orange-red complex $[Fe(phen)_3]^{2+}$ that is very stable in pH 3–4.5. An acetate buffer was prepared with 8.4 g of sodium acetate trihydrate and 0.1 M/L glacial acetic acid in 100 mL of DI water, and the final concentration of acetate solution was 6 M (pH 4.4). The 0.01 M 1,10-phenanthroline and 0.1 M hydroxylamine were prepared using the acetate buffer, respectively. Before the experiment began, 3 μL 0.1 M hydroxylamine and 3 μL phenanthroline were added to the detection area and air-dried in 10 min, respectively. Then 3 μL Fe sample was added using a micropipette and the color of the paper changed from white to red within 2 min. Finally, the paper was inserted to the device's sleeve directly and the corresponding signal intensity could be read out from the LCD screen directly.

2.4 Samples' measurements on μPADs

Here, we used the colorimeter to measure the reflected light. As well known, the black background has few light reflectance compared with the white one. Therefore, when the concentration increases, its color becomes deeper and its signal value for light reflectance decreases. I_B means the control value by measuring the blank white filter paper. I_R means measuring the reflectance values of the samples' colorimetric assay in our devices, respectively. And in every experiment, the final testing signal intensity I_T can be expressed by the Eq. (1).

$$I_T = I_B - I_R \tag{1}$$

The dynamic measurement range of the device is determined by the maximum and minimum intensities that are measurable for a white (full reflectance) and a black (no reflectance) strip. Every concentration of chemical or biological sample was measured for three times on three different strips for the subsequent experiments unless otherwise noted.

3 Results and discussion

3.1 Evaluation of quantitative performance for color sensitivity

In this approach, we employed the colorimetric paper-based assay by its reflectance light. The reflectance detection typically followed nonlinear formula as previous reports [1, 42, 47, 48] shown in the Eq. (2):

$$Y = A + B \times \log_{10}(X) \tag{2}$$

Before the analysis of the samples, the device's performance for color sensitivity was firstly evaluated by the standard pantone color paper. Because the pantone color matching system could standardize color in the CMYK (cyan, magenta, yellow, and black) process and provide more accurate color values. A group of pantone color papers were selected with the different value of M (from 20 to 80%) and the fixed CYK values (C = 100%, Y = 30%, K = 40%). Therefore, the color values of this group of pantone color papers were linear by testing by software ImageJ (data not shown). Then the pantone papers were directly inserted into the plastic sleeves and the corresponding color sensitivity was read-out from the device's screen. And as illustrated in Fig. 2A, a very good linearity with small errors was obtained ($R^2 = 0.996$) that proved that the device could be able to get good quantitative results of the color reaction. The novel portable device had some obvious advantages. The progress of using indexing oil to wet the paper by transmission method [43] was no longer needed. In addition, the paper's thickness was not associated with the signal because the reflectance light never passed through the

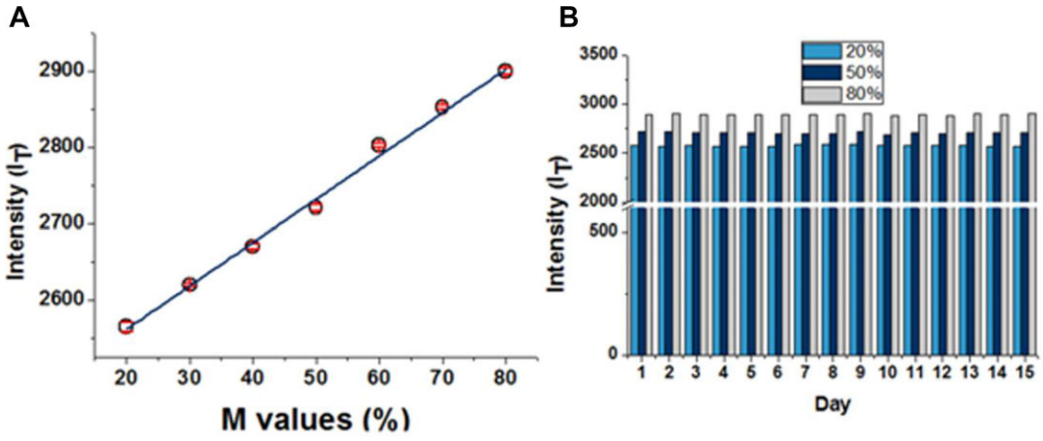

Figure 2. Colorimetric response of the pantone color papers with different *M* values in CMYK mode by letting the CYK values as constant ($C = 100\%$, $Y = 30\%$, $K = 40\%$). *M* values were selected from 20 to 80% (The RSDs are 0.69, 0.26, 0.47, 0.57, 0.40, 0.51, and 0.78%, respectively ($n = 3$)). The linear regression equation is $Y = 2447 + 5.69X$, $R^2 = 0.996$. (B) Measuring the signal of the pantone color papers with different values of *M* in CYMK modes in continuous 15 days. The 15 days' average value of strip 1, 2, 3 (strip 1, $M = 20\%$; strip 2, $M = 50\%$; strip 3, $M = 80\%$) showing good RSD that are 0.31, 0.33, and 0.27%, respectively ($n = 15$).

paper. Therefore, the whole test showed very small errors compared with light transmission method.

3.2 Signal stability in the μPADs

The device was used in the form of the detecting reflected light from the colorimetric assay. It was critical that diagnostic devices need keep stable during long time to meet the need of point-of-care and on-site settings. In order to characterize the stability for the signal the device, we used the pantone color papers with the different value of *M* (pantone paper 1, $M = 20\%$, paper 2, $M = 50\%$, paper 3, $M = 80\%$) to make observations of the signal in 15 continuous days. The testing environment was at room temperature under normal conditions. The main reason was to select pantone color paper because its color was stable and did not change with time that was very convenient to test the signal stability of our μPADs. The pantone color papers were directly inserted into the device. As illustrated in the Fig. 2B, it hardly found apparent signal difference between the different days for each pantone paper. The intensity of three testing papers with different *M* values was tested in 15 days. The average values for 15 days in different pantone paper were acquired and their RSDs were only 0.31, 0.33, and 0.27%, respectively. These results indicated that the changes in color intensity were obtained with very good reproducibility in our device.

3.3 Investigation for glucose and BSA assays

With the rapid development of science and technology, people need low-cost equipment to obtain accurate information on-site or resource-limited settings. To prove the concept and reliability of our μPAD device based on light reflectance principle that was capable of measuring the different types of biological colorimetric assays, the glucose as important biological indicator had been firstly investigated. The glucose was arranged at increasing concentrations from 3, 5, 8, 10, 20 to 30 mM for testing. In this case, the signal for the assay correlated with concentration of glucose analyte as illustrated in Fig. 3A and showed good linearity. The error bars indicated small SDs for three repeated measurements ($R^2 = 0.985$) and the LOD was 0.9 mM (calculated from 3σ). When the concentration was higher than 30 mM, the responses were strong but the linearity became worse (data not shown). This result was in agreement with previous literatures [49, 50].

Furthermore, the BSA assay was also implemented to characterize the capability of this device for potential bioassay applications as a model analysis. The results were displayed in Fig. 3B. As expected, the BSA detection zones exhibited gradually color changes with the increasing concentration. The patterned paper could be used to determine BSA concentrations in the range from 5 to 50 μM with satisfied results in our device and the LOD was 1.3 μM. When the BSA concentrations were more than 50 μM, the signal intensity reached a plateau and was not correlated with the concentration of the target well. And the linear regression equation was $Y = 2665 + 88.3 \times \log_{10}(X)$ ($R^2 = 0.974$). Compared with the BSA results in transmission method [43], it could quantify the concentration of BSA more accurately that indicate it was consistent with the discussion mentioned above.

3.4 Investigation for Fe (II) and nitrite assays

To illustrate the possibility of using our paper-based microfluidic device for the colorimetric assays of pollutants, Fe ion and nitrite were analyzed as models. 1, 10-Phenanthroline was chosen to test the concentration of Fe (II) in this device because it was known to be a good indicator for Fe (II) by forming the orange/red complex $[Fe(phen)_3]^{2+}$ and the whole

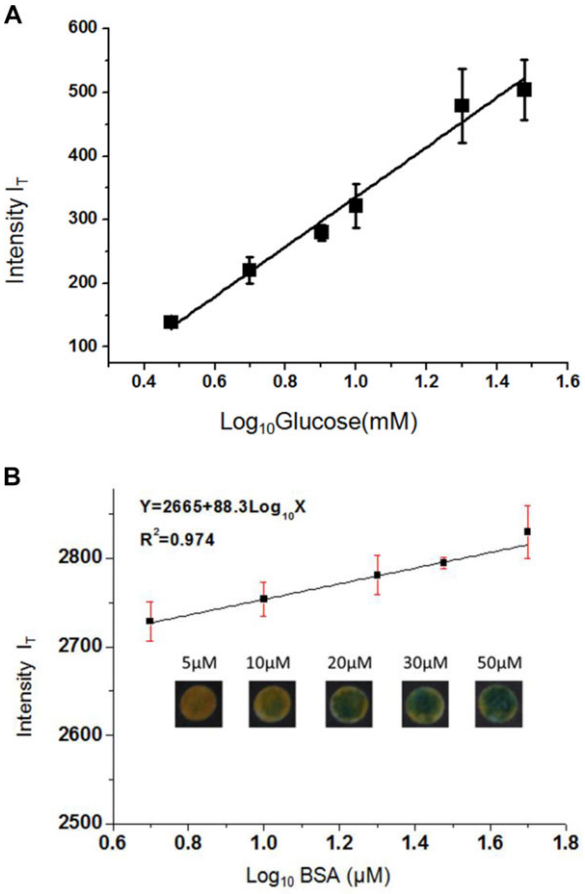

Figure 3. (A) The quantitative calibration curve of different glucose concentration (3, 5, 8, 10, 20, 30 mM) and its linear equation is $Y = 384.39 \times \text{Log10}(X) - 51.081$ ($R^2 = 0.985$). (B) Calibration curve for determination of the protein (BSA) using the portable device based on the light reflectance principle. The good response of this assay to the BSA concentrations is ranged from 5 to 50 μM ($R^2 = 0.974$).

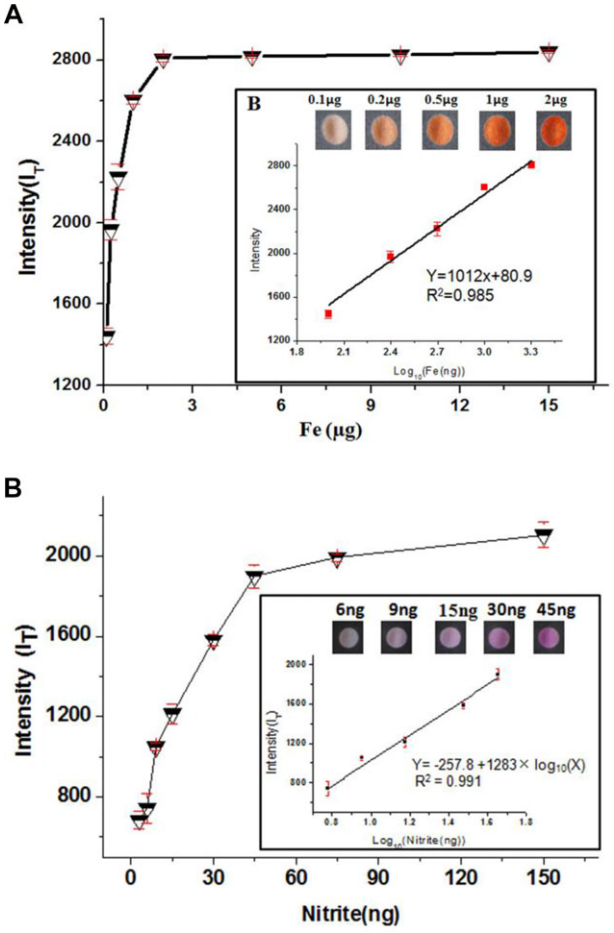

Figure 4. (A) Quantifying colorimetric Fe assays and signals were obtained using the hand-held reflectance colorimeter. The error bar in calibration curve represents SDs from three repeated experiments. Inset: the enlarged portion of the linear plot in the Fe (II) concentration range of 100–2000 ng. (B) Determination of nitrite with various concentrations on a colorimetric paper-assay format. When the amount of nitrite is more than 45 ng, the signal tends to be saturated. The inst was the calibration curve for the detection of nitrite.

mechanism was well understood. A 0.1 M hydroxylamine was added in Fe (II) solution to prevent Fe (II) oxidation to Fe (III). Furthermore, the hydroxylamine also functioned as a masking reagent to eliminate possible interfering metals Cd, Ni, Co, and Zn. Eight different concentrations of Fe (II) were added to the device. As illustrated in Fig. 4A, if the amount of Fe (II) was above 2 μg, the signal intensity will be saturated. And the enlarged portion of the plot showed a linear correlation with the concentration of the Fe (II). The dynamic linear range was determined from100 ng to 2 μg. The best-fit line was $Y = 1012 \times \log_{10}(X) + 80.9$, $R^2 = 0.989$. No noticeable change in signal intensity was observed above 2 μg of Fe (II). Because the on-site detection of environmental pollution is highly desired, this approach can be extended to more metal ions assay on-site monitoring by replacing 1,10-phenanthroline to their appropriate color indicators in this device.

To further demonstrate the concept of the colorimetric paper-based assay for the on-site detection of environmental pollution, we employed a nitrite as another model in this device. Nitrite was a characteristic pollutant, which was ubiquitous within water, soil, and food systems. It can react readily with secondary amine, tertiary amine, and amides in human body producing carcinogenic nitrosamines compounds. The NEDD chromogenic reagent was firstly introduced on the paper, after air dry, different concentrations of nitrite were added by micropipette and the reaction time was two minutes. As demonstrated in Fig. 4B, the intensity of the color was linearly dependent upon the amount of the nitrite (3 μL) with a linearity range from 6 to 45 ng that was equal to 2–15 mg/L. The LOD was calculated to 0.53 mg/L. When the amount of nitrite was above 45 ng, the signal intensity would be increasing slowly. These results clearly demonstrated the ability to discriminate between different concentrations of nitrite visually. The small SD for the measurements and the good linear relationship were obtained ($n = 3$). According to

the maximum nitrite containment level as recommended by United States EPA [39], it should be 3.29 mg/L (71.4 μM for NO_2^-) and our LOD (~0.53 mg/L) can satisfy this EPA standard. Besides, paper's capillary action provides a mechanism for separating particulates from the liquid [10]. Therefore, it has the basic ability of sample pretreatment such as filtering the suspended solid particles and impurities etc in our system. To some extent, this pretreatment characteristic renders the sample compatible with the assay and suitable to on-site detection without other external equipment operations. Moreover, there is still an urgent need and challenge to obtain a user-friendly, higher sample pretreatment performance by the development of the paper-bases microfluidics technology.

3.5 Recovery of Fe (II) and nitrite samples

In order to show the performance and reliability of our device, the recovery of the Fe (II) and nitrite samples of local tap water were performed by our method and standard UV absorption method (shown in Supporting Information Table 2). The reason for not selecting the BSA and glucose was because that their real samples were relatively difficult to obtain. The quantitative curves for Fe (II) and nitrite samples using standard UV absorption method were shown in Supporting Information Figs. 2 and 3, respectively. The recovery ranges of the spiked nitrite and Fe (II) samples were 99.3–105.2% and 94.0–108.6%, respectively, in our device with small deviations. Moreover, to further verify the reliability of the results obtained from our device, the UV method to analyze the same nitrite and Fe (II) samples. As displayed in Supporting Information Table 2, the recovery of Fe (II) and nitrite were 99.0–102.9% and 96.8–103.5%, respectively. There were little differences from the results obtained from our μPAD device and ultraviolet absorption instrument. Though the recovery range in our device was little wider than the UV method, considering the ability of easy use and transportation, our device could provide a more rapid, quantitative, and user-friendly way to realize the on-site or resource-limited detection.

4 Concluding remarks

In conclusion, a novel kind of portable μPAD device based on light reflectance was proposed. In addition, compared with the previous method by measuring the light's transmission from the strip, to our knowledge, the reflectance-based μPAD is firstly presented. This portable μPAD could provide real-time quantitative information from colorimetric assays. The approaches for the determination of nitrite, Fe, and protein had been investigated and showed good performance with reproducible results. And the successful analysis of the real samples of Fe and nitrite in the local water showed good sensitivity and reproducibility. In comparison with the results obtained from our device and the ultraviolet absorption method, there were no noticeable differences that indicated the reliability of the μPAD device was verified and trusted. In addition, the different kinds of metals or pollution assays on-site monitoring could be implemented through using their appropriate color indicators and would show great potential and versatility to on-site detection.

Future work includes reducing the power consumption, regulating the lighting conditions, and performing simultaneous measurement of a number of different colorimetric reactions with high throughput detection. Briefly, combining the promising advantages of feasible, easy transportation, and manipulation with cheap price, this device will also have potential to be applied to obtain quantitative data from the fields of on-site detection, medical diagnostics, and environmental pollution evaluation.

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