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An Automated Microfluidic Paper-Based Analytical Device for Chemiluminescence Immunoassay

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***Abstract*— This paper introduces a high-integrated microfluidic paper-based analytical device (μPAD) that has a reliable and programmable rotary valve and automated injection device. By controlling the rotation of valves, different regions on the μPAD can be connected or disconnected, allowing reagents to be effectively transported to the test zone. In order to address the limitations of traditional chemiluminescent immunoassays (CLIA), which involve expensive equipment and extensive manual operations, we utilize a smartphone to read the results and program each component of the device to automate the detection process. As a proof-of-concept, we conducted the detection of rabbit IgG under optimized experimental conditions (H_2O_2 concentration at 0.1 M, HRP-conjugated antibody concentration at 150 μg/mL, and plasma treatment time of 4 minutes), achieving a limit of detection of 3.58 pM. Our device combines the multifunctionality of μPAD with the sensitivity and specificity of CLIA, which potentially advances the development of point-of-care testing.**

Keywords—Microfluidic paper-based analytical devices, Chemiluminescence immunoassay, Point-of-care testing

I. Introduction

In recent years, point-of-care testing (POCT) has received much attention due to its important role in healthcare diagnostics. Among the emerging technologies for POCT, microfluidic paper-based analytical devices (μPADs) have garnered increasing recognition as promising tools. Initially proposed by Prof. Whitesides in a paper-based format, μPADs offer unique advantages for highly selective analysis in complex biological samples [1-4]. These devices are characterized by their cost-effectiveness, sensitivity, specificity, and reliability, making them particularly attractive for clinical diagnostics.

Several analytical methods have been successfully developed on μPADs, including colorimetry [5], fluorescence [6], chemiluminescence (CL) [7, 8], electrochemistry [9], and electrochemiluminescence [10]. Among these methods, CL stands out as one of the most potential techniques due to its low limit of detection (LOD), high reproducibility, wide linear range, and fast response [3, 7]. Unlike colorimetry or fluorescence, CL does not require an external light source for excitation and is unaffected by the color of the matrix [11]. A common practical application of CL is the luminol-hydrogen peroxide (H_2O_2)-horseradish peroxidase (HRP) reaction system, where HRP is commonly used to label antibodies [12]. However, CL requires expensive cooled charge-coupled device cameras or photomultiplier tubes (PMT) for reading [13], which goes against the advantage of the low cost associated with POCT.

Smartphones present potential alternatives due to their associated components, such as high-resolution metal-oxide-semiconductor cameras and powerful computing capabilities, making them potentially suitable for scientific applications [8].

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The availability, affordability, and portability of smartphones make them a practical alternative to conventional laboratory equipment for detection purposes. By simplifying readout strategies and minimizing detection requirements, coupled with advancements in smartphone camera sensors towards higher light sensitivity, they offer a viable solution for CL measurements without the need for expensive and high-maintenance photodetectors [8, 11, 13-15].

Several platforms for performing chemiluminescence immunoassay (CLIA) on μPADs have been reported, each with its sensitivity and rapid testing capabilities. Chen *et al.* developed a paper-based CLIA device enhanced by carbon nanosphere signal amplification. They synthesized hollow carbon spheres as nano-carriers functionalized with HRP [16]. However, CLIA signal collection requires a PMT associated with an ultra-weak luminescence analyzer, significantly increasing costs and reducing portability. Shang *et al.* reported a paper-based microfluidic CL device driven by both gravity flow and capillary forces, with CL intensity captured by a low-cost charge-coupled device [17]. Notably, their device requires users to add luminol solutions quickly and manually to the paper-based loading area using a pipette. Moreover, after addition, the device's aperture needed swift closure to eliminate environmental light interference with the CL signal. Lazzarini *et al.* developed a paper-folding-based CLIA device where reagents are preloaded in dry form on the μPAD, and all procedures were conducted by folding or unfolding the device [18]. However, manual folding or unfolding during the immunoassay steps introduced potential errors. Therefore, these CLIA studies inevitably involve manual operations, such as pipetting or manipulating μPADs, potentially introducing errors and additional training costs for users [17-21].

In this study, we introduce an automated μPAD featuring a reliable rotary valve system. This device is characterized by a highly integrated injection system capable of accurately introducing pre-mixed reagents, including luminol, catalysts, and enhancers, at precise intervals during the detection process to initiate CLIA. The rotary valve can connect or disconnect microchannels on the μPAD by simple rotating. To obtain the results, we used a smartphone-based detection method, which is user-friendly and operable even by untrained personnel. Captured images are subsequently analyzed and processed via PC software. We employed the device to conduct an enzyme-linked immunosorbent assay (ELISA) to measure the concentration of rabbit IgG in a phosphate-buffered saline (PBS) solution (N=5), yielding an LOD of 3.58 pM and a coefficient of determination (COD, denoted as R^2) of 0.997. This demonstrates greater sensitivity compared to the colorimetry (20 pM) [1], and comparable performance to CLIA using magnetic nanoparticles, which have an LOD of 4 pM [22].

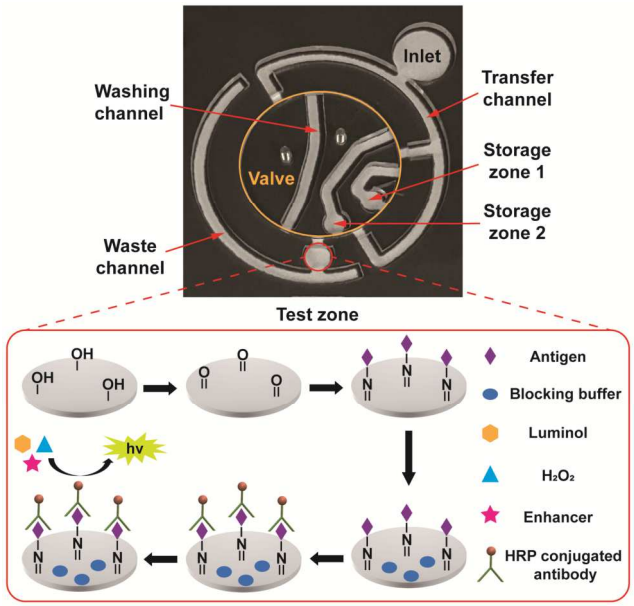

Fig. 1. The photo of developed μPAD consisted of a rotary valve and a surrounding chip with the protocol of CL ELISA in the test zone.

II. METHODS

A. Design and Function of the μPAD

Fig. 1 illustrates the design of our μPAD used for CLIA based on the protocol of ELISA. The μPAD primarily comprises a rotary valve and a surrounding paper-based chip. The chip consists of hydrophobic areas created through wax printing, along with hydrophilic areas. A hydrophobic layer fully covered by wax is tightly bound to control liquid flow below the chip. In addition, a polymethyl methacrylate (PMMA) case is fixed beneath the chip layer to improve robustness and stability [5]. The rotary valve layer primarily consists of three channels, including a washing channel and two channels connecting to the storage zones. Meanwhile, the chip layer features an inlet, a waste channel, and a test zone. Two storage zones within the valve store the reagents required for CLIA. During the experiment, PBS is first added into the inlet and flows into the transfer channel. The liquid in the channels then washes the pre-stored reagents, which are in dry form within the storage zones, into the test zone. Through the rotation of the valve, various channels or storage zones can be sequentially and programmably connected with the reaction zone. By rotating for connecting, the liquid flows out of the flushing channel into the waste channel through the test zone.

B. Fabrication Process of the μPAD

The channels on the μPAD were designed using AutoCAD software (Autodesk Inc., U.S.A.) and printed onto Whatman No. 4 chromatography paper using a wax printer (8580DN, Xerox). Subsequently, the paper was heated for 120 seconds at 120 ℃ on a hotplate (CS15956-31, Cole-Parmer) to melt and penetrate the wax into the paper, forming a hydrophobic barrier. After cooling to room temperature, the paper was cut into the designed shape using a laser cutter (CMA0604-B-A, Han's Yueming), and the PMMA case was wrapped around it to package the μPAD. For protein immobilization in the reaction area, the paper underwent plasma treatment for 4 minutes using a plasma treatment device (VP-R3, SUNJUNE, China) [23], with a frequency of 13.56 MHz and a power of 100 W, resulting in the formation of aldehyde groups in the reaction area. Then, 3 μL of rabbit IgG (I5006, Sigma-Aldrich, Shanghai, China) in PBS (G4207, Servicebio, Wuhan, China)

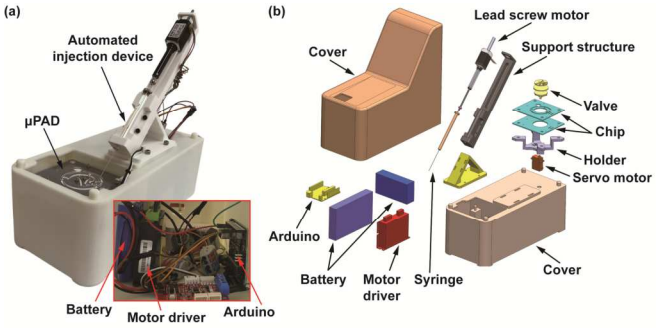

Fig. 2. (a) The photo of the developed device and its internal components. (b) Exploded diagram of the entire system.

was added and incubated for 20 minutes. Additionally, 3 μL of HRP-conjugated IgG antibodies (SE134, Solarbio, Beijing, China) in PBS and 3 μL of blocking buffer (0.1% (w/v) bovine serum albumin (BSA, G5001, Servicebio, Wuhan, China) in PBS) were added to the printed storage zones on the valve before the experiment began and dried for 20 minutes at room temperature. A pre-mixed solution of 1 mM luminol (L105655, Aladdin, Shanghai, China), H_2O_2 (10011208, Sinopharm, Shanghai, China), and 3 mM enhancer (4'-(1H-1,2,4-triazol-1-yl) phenol, 1214269, Leyan, Shanghai, China) was prepared and loaded into a syringe, which was then injected into the reaction zone. The pre-mixed solution is stored light-protected at 4 °C, and testing has shown that it does not significantly affect the experimental results for up to three days. Moreover, the liquid in the syringe and the paper-based chip are replaced after each detection.

C. Automated μPAD integrated with a Smartphone

The automation of the device relies on the integration of several components, including a microcontroller (Arduino UNO R3), a micro servo motor (SCS2332, Feetech), a lead screw motor (20K-2M-50.8, Yingpeng Aircraft Electric, Shenzhen, China), and a syringe, as shown in Fig. 2(a). The servo motor drives the rotation of the paper-based rotary valve, thereby regulating the entire CLIA process. Meanwhile, the led screw motor, controlled by the microcontroller, automatically pushes the syringe, injecting the pre-mixed solution into the test zone. A slot positioned directly above the μPAD is designed to hold a smartphone (Apple iPhone 13), which captures a 30-second exposure photograph to collect the CL results. To support these components, a support structure and covers, as shown in Fig. 2(b), were fabricated using a 3D printer (S5 Pro Bundle, Ultimaker) with tough white polylactic acid. The overall device size is 140 × 280 × 300 mm, and the paper-based chip size is 78 × 89 mm, with a test zone radius of 3 mm and a rotary valve radius of 15 mm. Moreover, the distance from the smartphone camera to the test zone is 10 cm.

III. Results

As a proof-of-concept, we performed a direct ELISA (N=5) on our automated μPAD, with concentrations of rabbit IgG ranging from 6.7 pM-6.7 μM in PBS solution. Remarkably, lower concentrations of reagents correspond to reduced luminescence intensity at each antigen concentration, which

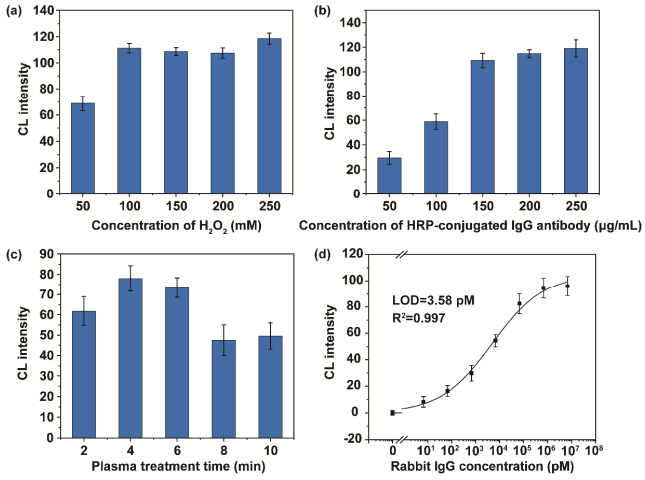

Fig. 3. (a) CL intensity vs concentration of H_2O_2 from 50 mM to 250 mM. (b) CL intensity vs concentration of HRP-conjugated IgG antibody from 50 μg/mL to 250 μg/mL. (c) CL intensity vs oxygen plasma treatment time from 2 mins to 10 mins. (d) Calibration curve of CL intensity vs rabbit IgG concentration (N=5).

reduces fluctuations in light intensity across different concentrations. Moreover, high concentrations of reagents may increase background noise and reduce sensitivity. Therefore, determining the optimal reagent concentration is essential for the system's accuracy, sensitivity, and reliability.

Fig. 3(a) presents the CL intensity obtained at different H_2O_2 concentrations, with all results measured under a fixed HRP concentration of 100 μg/mL and a rabbit IgG concentration of 6.7 μM. The intensity peaks at an H_2O_2 concentration of 0.1 M. And further increases in concentration result in a decrease. Therefore, 0.1 M is selected as the optimal concentration.

The concentration of HRP is another factor affecting CL intensity. Fig. 3(b) illustrates the impact of varying HRP concentrations on CL intensity. All results were obtained under a constant H_2O_2 concentration of 0.1 M and a rabbit IgG concentration of 6.7 μM. It was found that a concentration of 150 μg/mL resulted in higher and stable CL intensity. CL intensity changes largely when the concentration is lower than 150 μg/mL and increases slightly when the concentration is higher than 150 μg/mL. Therefore, 150 μg/mL was chosen for subsequent experiments.

The influence of oxygen plasma treatment time on the antibody immobilization process has been previously investigated [23]. Excessive processing time can lead to the destruction of the fiber structure, resulting in a messy floc that is not conducive to antibody immobilization. Therefore, finding the appropriate treatment time is essential. As shown in Fig. 3(c), variation in CL intensity under different oxygen plasma durations is presented. The results indicate that the highest CL intensity is achieved at 4 minutes of treatment time.

The platform and the valve were pre-programmed according to the CL protocol, comprising several steps: Firstly, PBS is added into the inlet as a washing solution, flushing the blocking buffer from the storage zone into the test zone,

followed by a 5-minute incubation. Secondly, the HRP-conjugated rabbit IgG antibody is flushed into the reaction zone and incubated for 5 minutes. Thirdly, PBS flows through the reaction zone from the washing channel, rinsing the non-specifically bound antibodies and BSA into the waste zone. Fourthly, the premixed CL solution is injected into the reaction area, and the results are captured using a smartphone camera. The resulting images are imported into FIJI (ImageJ) and then fitted with Hill's equation using Origin software (OriginLab, U.S.A.). The grayscale value of the test zone is calculated to reflect the CL intensity. Each antigen concentration undergoes five replicates. The calibration curve achieves an LOD of 3.58 pM and an R^2 of 0.997 (Fig. 3(d)). No valve malfunction occurs during the repeated tests, further proving the reliability and stability of the device. The sensitivity is higher than the colorimetric methods (20 pM) and CLIA using magnetic nanoparticles (4 pM) [1, 22], further confirming the application potential of this device.

IV. Conclusion

This work introduces an automated μPAD for CLIA, combining the simplicity and low cost of μPADs along with the sensitivity and high selectivity of CL. The integration of a smartphone enables low-cost and user-friendly data collection, distinguishing it from traditional POCT devices. Designed especially for CLIA, this device allows for automatic detection, reducing the need for manual operation. A reliable and automated rotary valve is integrated to store the required reagents for CL and control fluid flow through rotation. Additionally, the device successfully detected varying concentrations of rabbit IgG using direct ELISA, achieving an LOD of 3.58 pM, which is more sensitive compared to other methods. We believe that this device holds significant potential for healthcare improvement in developing countries or resource-limited areas due to its integration, reliability, low cost, and ease of use. Furthermore, it potentially advances the development of POCT.

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