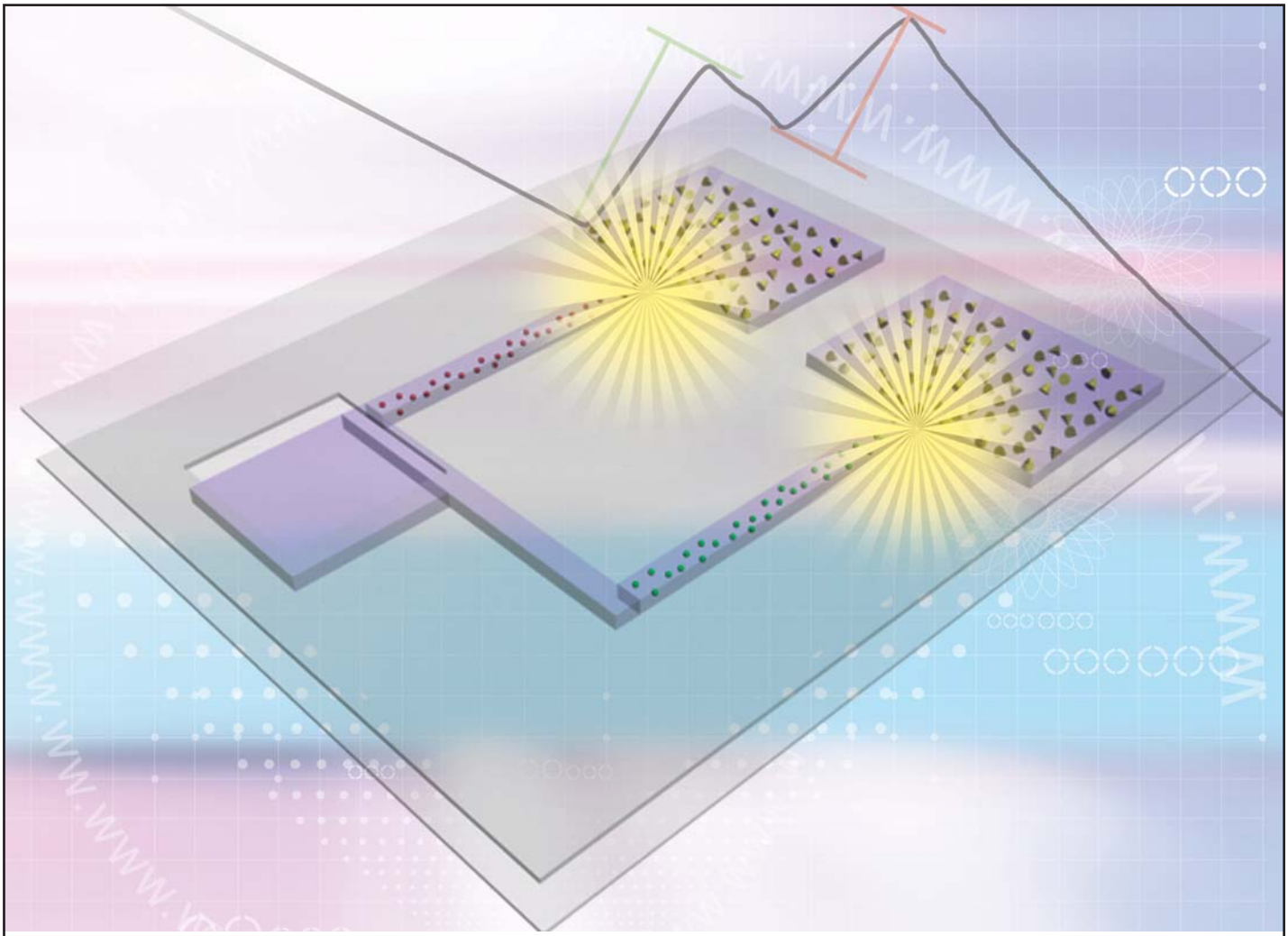

Featuring research from the groups of Prof. Dr Jinghua Yu at the University of Jinan, School of Chemistry and Chemical Engineering and Prof. Dr Jiadong Huang at the University of Jinan, College of Medicine and Life Sciences, Jinan, China.

Title: Microfluidic paper-based chemiluminescence biosensor for simultaneous determination of glucose and uric acid

The development of a chemiluminescence method on wax-patterned microfluidic paper-based device in this work expands the lab established on paper. The principle and multichannel application of the chemiluminescence method on this device has been proved.

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PAPER

Microfluidic paper-based chemiluminescence biosensor for simultaneous determination of glucose and uric acid†

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In this study, a novel microfluidic paper-based chemiluminescence analytical device (μPCAD) with a simultaneous, rapid, sensitive and quantitative response for glucose and uric acid was designed. This novel lab-on-paper biosensor is based on oxidase enzyme reactions (glucose oxidase and urate oxidase, respectively) and the chemiluminescence reaction between a rhodanine derivative and generated hydrogen peroxide in an acid medium. The possible chemiluminescence assay principle of this μPCAD is explained. We found that the simultaneous determination of glucose and uric acid could be achieved by differing the distances that the glucose and uric acid samples traveled. This lab-on-paper biosensor could provide reproducible results upon storage at 4 °C for at least 10 weeks. The application test of our μPCAD was then successfully performed with the simultaneous determination of glucose and uric acid in artificial urine. This study shows the successful integration of the μPCAD and the chemiluminescence method will be an easy-to-use, inexpensive, and portable alternative for point-of-care monitoring.

Introduction

Seemingly, there are two opposite trends in current non-invasive clinical diagnostics: one toward extensive automation and consolidation of testing in central laboratories, another toward portable point-of-care (POC) diagnostics and on-site detection.[1] The frequency of adult disease is increasing due to the increasingly aging population, which increases the cost of health care. Therefore, simple and low-cost methods that can be used at home to monitor component indices are required to detect the onset of diseases before serious complications arise. Although costs and assay times in central laboratories have recently been significantly reduced, POC is preferred when test results are needed more rapidly and conveniently. Lab-on-a-chip, where channels, pumps and valves are created on a plastic (or glass, silicon) substrate, is currently the most popular POC diagnosis device. Its importance and utility are widely acknowledged and extensive research has been conducted in the laboratory on device manipulation and proof-of-concept demonstration, but it has not yet become widely used, particularly by those in developing countries[2] due to the complex fabrication, expert requirement and expensive components.

Recently, much effort has been directed toward the development of simple, low-cost, practical diagnostic tools that are amenable to POC diagnosis such as rapid screening of specific target analytes in the health, food, and environment sectors.[2–9] Paper, being relatively cheap, abundant, sustainable, disposable and easy to use, store, transport, and modify, is already used extensively as a platform in analytical and clinical chemistry.[10] The first paper-based POC diagnosis device was a paper strip which dates back to the early 20th century, and a big breakthrough was the invention of paper chromatography for which Martin and Synge were awarded the Nobel Prize in chemistry in 1952. The most typical examples are immunochromatographic tests[11] such as the well-known pregnancy test strip. These paper strip tests are advantageous because of their simplicity and low-cost, but often suffer from the fact that they are only "yes/no" detections, not quantitative, and lack the ability for multiplex analysis.[11,12]

To address these disadvantages, the newly developed microfluidic paper-based analytical devices (μPADs), which combine the simplicity and low-cost of paper strip tests and the complexity of the conventional lab-on-chip devices, are very attractive[13–24] and hold a lot of potential for POC and on-site diagnosis. Paper is a porous cellulose fiber web with a high surface area; this porous nature not only fulfils the primary tasks of any diagnostic tests using body fluids and fluid transport, but also means it can be patterned into channels of hydrophilic surfaces separated by hydrophobic walls of photoresist/polymer,[15,20,21,25] inks,[19] wax[26,27] and plasma treatment [22] or by cutting method.[17] Currently, only a few test methods are established on μPADs, such as colorimetric[13–15] and electrochemical methods.[16,28–30] Absorbance and fluorescence methods have been proved to be potential test methods on μPADs.[21] Chemiluminescence (CL) has become an attractive method in

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biosensors because of its simplicity, high sensitivity, low cost, low-power demands, and high compatibility with micromachining technologies.[31] To the best of our knowledge, no reports about establishing CL biosensors on μPADs have been published. In this paper, we demonstrate a novel rhodanine derivative CL system between a rhodanine derivative and generated hydrogen peroxide in Tris-HCl buffer based on the long-term basic research on rhodanine derivative CL systems in our lab,[32–35] and found that this CL system has a well-defined CL response on this μPAD. In this work, the rhodanine derivative 3-*p*-nitrylphenyl-5-(4′-methyl-2′-sulfonophenylazo) rhodanine (M4NRASP) is used as a model rhodanine[35]

The simultaneous quantification of glucose and uric acid in urine has great clinical importance. Glucose levels can be related to the diagnosis of diabetes, alcohol consumption, obesity and high cholesterol,[36] while uric acid levels can be related to the diagnosis of gout, high blood pressure, kidney disease and heart disease[37–41] and so on. In recent years, a number of studies have been conducted to develop new glucose monitoring methods[42–45] and new uric acid monitoring methods.[36,46–49] However, these methods require expensive, complicated instrumentation or operating steps and can generally only be done in the laboratories. The purpose of this work is to develop a novel CL enzyme biosensor based on a lab-on-paper device, which pursues portable sensing, for the fast, simple and simultaneous quantification of glucose and uric acid. Combining the newly demonstrated cut-patterned μPADs[17] and rhodanine derivative CL system, a novel multiplex microfluidic paper-based chemiluminescence analytical device (μPCAD) biosensor was established based on facile enzyme/reagent immobilizations and cut pattern technology. This cut pattern, which is never exposed to photoresists or other polymers and inks that could contaminate them or interfere with the CL and enzyme reaction on the μPCAD, will be more suitable to fabricate μPCAD biosensors. The possible CL assay principle of this μPCAD biosensor is explained. We found that the simultaneous quantification of glucose and uric acid could be achieved by differing the distances that the glucose/uric acid samples traveled. This novel method has simpler operation, higher sensitivity, better selectivity and requires smaller sample volumes and shorter response times. Finally, this μPCAD biosensor was applied to the simultaneous quantification of glucose and uric acid in artificial urine.

Materials and methods

Reagents and materials

All reagents were of analytical reagent grade or above and used as received without further purification. M4NRASP was obtained from our lab and dissolved in anhydrous alcohol.[35] Glucose oxidase (GOx) (E.C. 1.1.3.4 from *Aspergillus niger*; 185 000 U g^{-1}) and urate oxidase (UOx) (E.C. 1.7.3.3 from *Arthrobacter globiformis*; 18 000 U g^{-1}) were purchased from Sigma-Aldrich (Tianjin, China). Uric acid and D-(+)-glucose were purchased from Sigma-Aldrich (Tianjin, China). Whatman chromatography paper #1 (WCP#1) (200.0 mm × 200.0 mm) was obtained from GE Healthcare Worldwide (Pudong Shanghai, China) and used with further adjustment of size. We chose this type of WCP because of its uniform composition (relative to other types of paper) without any additives that affect flow rate and CL reaction. Oxygen-saturated Tris-HCl buffer solution (TBS) (pH = 6.4) is used in the experiments for enzyme–substrate reaction and CL determination.

Fabrication and design of the paper device

A schematic representation of the stacked, alternating layers of patterned WCP#1 and patterned single-sided adhesive tape is shown in Fig. 1. This μPCAD was fabricated by stacking one layer of assembled WCP#1s (patterned in ways that channel the flow of fluid within the paper) between two layers of water-impermeable single-sided adhesive tape. The size of each component is shown in Fig. 2. A cutting method was used to pattern the middle assembled WCP#1 layer according to the previously reported method.[17] They were patterned using a computer controlled X-Y knife plotter (Graphtec FC7000-75, Shenzhen Honghui Advertisement Technology Co., Ltd., China) through a three sequential overlapping cuts method to avoid the tearing of the WCP#1. The first two sequential cuts penetrate only part way through the WCP#1. Following cutting operations, the removal of unwanted material to generate the patterns was performed manually. The middle assembled WCP#1 layer of this μPCAD was comprised of a sample injection area (sizes are shown in Fig. 2), two respective bioactive channels (8.0 mm × 0.5 mm) and two respective CL detection areas (4.0 mm × 3.0 mm). The sample injection area was pure WCP#1 without any immobilization. Bioactive channels were prepared by immobilizing enzymes on the WCP#1 channels. The enzymes were immobilized by a simple adsorption technique into the porous structure of the WCP#1 channels. Immobilization was carried out in batch by immersing the WCP#1 channels (ten channels each time in this study) into concentrated enzyme solution at 4 °C for 5 min. After this period, enzyme immobilized WCP#1s were removed from the enzyme solution and dried in a freeze-drying box. CL detection areas were prepared in batch by immersing the rectangular WCP#1s (ten papers each time in this study) into the M4NRASP anhydrous alcohol solution for 5 min, and dried at room temperature in air. Then, briefly, the middle WCP#1 layer of this μPCAD was firstly attached and assembled onto the bottom tape layer (20.0 mm × 15.0 mm), and then it was covered by another tape layer, which was patterned to form a square hole (3.0 mm side length) in it, to seal the WCP#1 layer. The pattern for the hole was designed with Adobe Illustrator software (Adobe Systems, Inc.) and fabricated by a laser cutter (Universal Laser VL-300 50 Watt Versa Laser). The hole in the top-tape should be aligned to sample injection area. These two steps can be finished within 10 min and will produce the cut-patterned paper devices.

Chemiluminescence assay procedure of this μPCAD biosensor

As shown in Fig. 2, the CL signal was measured using a computerized ultraweak luminescence analyzer (Type RFL-200, manufactured at Xi'an Remex Analysis Instrument Co, Ltd, Xi'an, China). There was a newly designed device-holder (20.0 mm × 15.0 mm) at the bottom of the cassette to fix the μPCAD. And the cassette can be shut with a black metallic cover which had an injection hole for sample injection. When the μPCAD was

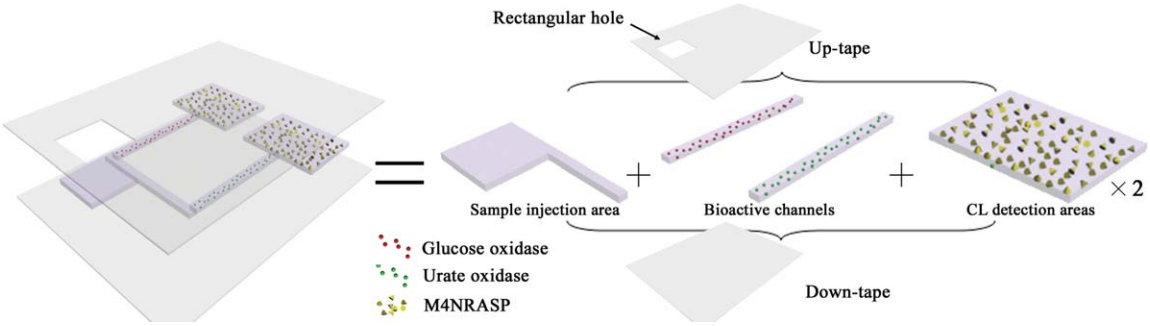

Fig. 1 Schematic representation of the fabrication of the μPCAD.

put into the holder, the sample injection area and CL detection areas were aligned exactly to the injection hole and the photomultiplier of the analyzer, simultaneously. For the CL assay, 30 μL of sample solution containing a desired concentration of glucose and uric acid in TBS was dropped onto the sample injection area through the hole by a pipette. The sample solution was migrated through bioactive channels toward the CL detection areas to obtain a CL signal. The CL signal was recorded using a computer. Data acquisition and treatment were performed with RFL software running under Windows 97. The concentration of sample was quantified by the peak height of the CL signal.

Results and discussion

Chemiluminescence assay principle of μPCAD

As mentioned above, this μPCAD is composed of one sample injection area, two respective bioactive channels and two respective CL detection areas (Fig. 1). All the components were assembled between two water-impermeable single-sided adhesive tapes. For the CL assay, as a model, 30 μL of sample solution containing 15.0 mmol L^{-1} glucose or 15.0 mmol L^{-1} uric acid in TBS was dropped onto the sample injection area as shown in Fig. 2. Subsequently, the analytes migrated along the porous WCP#1 by capillary action and then reacted with the GOx or UOx on the bioactive channels according to the specific enzyme–substrate reaction. The generated hydrogen peroxide and TBS buffer solution continued to migrate along the bioactive channels, governed by the Lucas–Washburn equation.[50] Then the hydrogen peroxide contacted with the CL detection areas at the adherent point with a relatively high fluid velocity. Therefore, the reaction between the hydrogen peroxide and preloaded rhodanine derivative in TBS was kinetics controlled and conducted immediately. This resulted in the remarkable CL emission simultaneously and a steep peak was obtained (Fig. 3A). As the subsequent hydrogen peroxide continued to flow into the expansive CL detection areas, the fluid velocity on the CL detection areas decreased remarkably and the Lucas–Washburn dynamics were not suitable.[50] Thus the CL reaction changed to diffusion control and the CL signal stopped increasing due to the depletion of the reactants in the advancing liquid front. In addition, the continuous decreasing of the fluid velocity decreased the concentration of hydrogen peroxide that contacted the CL reagent partly due to the loss of the hydrogen peroxide, thus the CL signal went down slowly (Fig. 3A). Quantitative analysis could be realized by reading the peak height. The more analyte in the sample, the more hydrogen peroxide would be generated into the CL detection areas, which leads to the increase

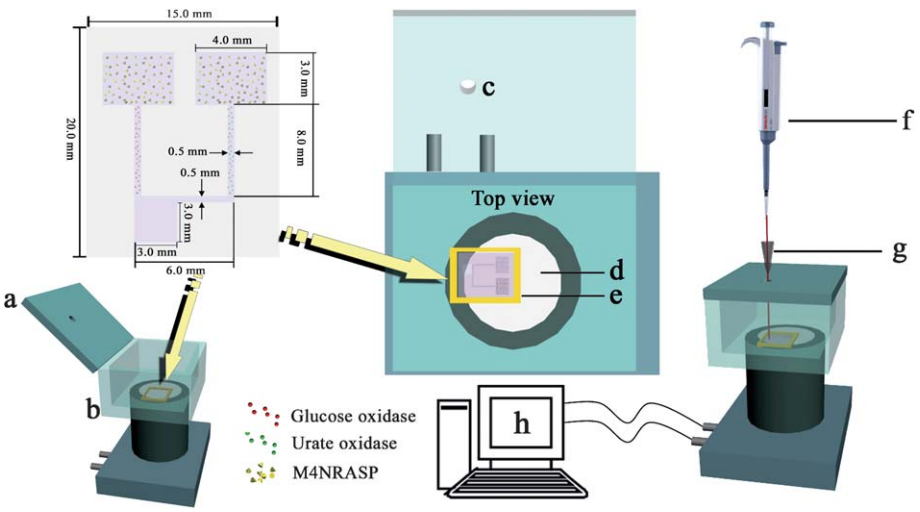

Fig. 2 Schematic diagram and assay procedure of this μPCAD biosensor: (a) black metallic cover; (b) cassette; (c) injection hole; (d) photomultiplier; (e) device-holder; (f) pipette; (g) rubber seal cone; (h) ultraweak luminescence analyzer.

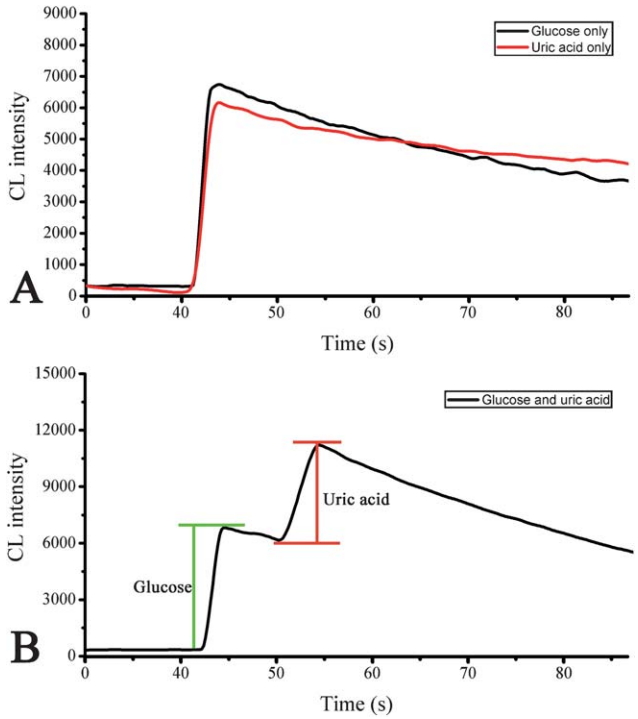

Fig. 3 CL responses for this μPCAD biosensor: A, independent responses of glucose and uric acid; B, simultaneous response of glucose and uric acid.

in CL intensity. According to the principle described above, the CL intensity on the CL detection areas would be proportional to the concentration of glucose and uric acid in the samples. The typical corresponding CL simultaneous response of this μPCAD to glucose and uric acid is shown in Fig. 3B. The position of the sample injection area results in different distances that the sample solution travels, thus the CL reaction would be conducted at different times and the CL responses of the glucose and uric acid would be separated obviously.

Optimization of the μPCAD and CL system

According to Darcy's Law,[50] the width of the bioactive channels was designed as narrowly as possible to decrease the reagent requirement and increase the fluid velocity. Considering the actual resolution of the knife plotter and the properties of WCP#1, 0.5 mm was selected as the final width of bioactive channels. In this μPCAD biosensor, TBS was used as the carrier solution. Therefore, the influence of the pH value of TBS on the enzymatic reaction activity and the generated CL signal was an important factor that determined the overall response of this μPCAD biosensor. In view of the nature of the traditional rhodanine derivative CL reaction, which is more favored under acid conditions,[32,33] an acid medium would improve the sensitivity of the system. However, the optimal pH value for UOx is 7[51] and GOx is 5–7.2.[52,53] Thus, the effect of medium pH of TBS on the biosensor was investigated through changing the pH value of TBS from 4 to 8. Using the ultraweak luminescence analyzer, the CL intensity reached the highest value at pH 6.4. Therefore, this μPCAD biosensor assay was performed at pH 6.4 throughout this study.

As the CL reagent in this device, the amount of loaded M4NRASP on the CL detection areas directly affects the CL response of this μPCAD biosensor. To investigate the optimal amount of M4NRASP for the CL assay, the CL detection areas were immersed into various concentrations of M4NRASP in the range of 50.0 to 100.0 mmol L^{-1} for 5 min. The result showed that the optimal concentration of M4NRASP was 72.0 mmol L^{-1} to save the reagent. The amount of enzyme, loaded on the bioactive channels by physical absorption, also affects the CL sensitivity of this μPCAD biosensor. To probe the optimal amount of the absorbed enzyme for the CL assay, we diluted the enzyme solution into various concentrations and investigated the influence on the signal-to-noise (S/N) ratio of the biosensor for 15.0 mmol L^{-1} glucose and uric acid respectively. As shown in Fig. 4, the S/N ratio was found to be highest for dispensing 0.4 g mL^{-1} GOx and 0.37 g mL^{-1} UOx for 5 min, respectively. The decrease in S/N at higher concentrations resulted from the increase in background signal due to too high a concentration of enzyme while that at lower concentrations is ascribed to the decrease in signal due to too low an amount of enzyme. Therefore, 0.4 g mL^{-1} GOx and 0.37 g mL^{-1} UOx were routinely used as the optimal concentrations of enzyme solutions throughout the entire study. In addition, too high an amount of enzyme may block the porous structure of the WCP#1 and decrease the capillary action which decreased the efficiency and sensitivity of this biosensor. All the other device sizes and shapes were designed to decrease the reagent requirement as much as possible.

Analytical performance of this μPCAD biosensor for glucose and uric acid measurement

Under the optimal conditions, Fig. 5A shows the typical responses of this μPCAD biosensor within 2 min for glucose/uric acid with different concentrations of 2 mmol L^{-1}/47 mmol L^{-1}, 10 mmol L^{-1}/10 mmol L^{-1}, 30 mmol L^{-1}/5 mmol L^{-1} and 50 mmol L^{-1}/2 mmol L^{-1}, respectively. As shown in Fig. 5A, well-defined curves were observed in the presence of glucose and uric acid, and the peak heights were getting higher/lower as the

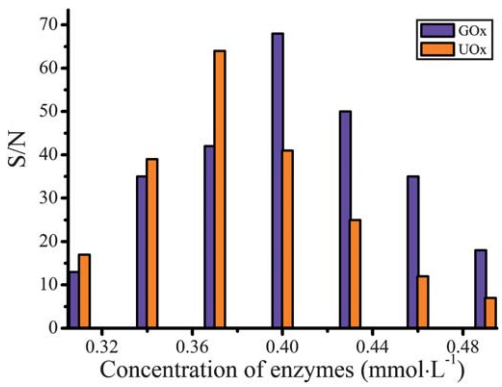

Fig. 4 Effect of the concentration of enzyme solutions on the signal-to-noise ratio (S/N) of the μPCAD for 15.0 mmol L^{-1} glucose and uric acid respectively.

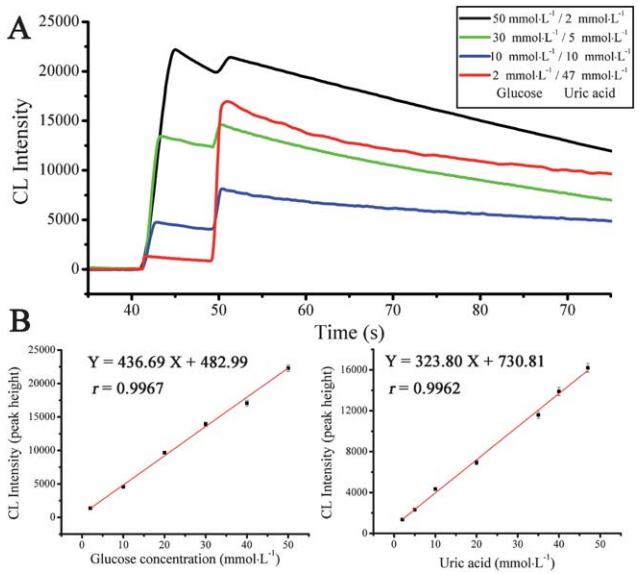

Fig. 5 Simultaneous and quantitative CL responses of glucose and uric acid with different concentrations. The linear regression equations for simultaneous determination of glucose and uric acid are shown in the inset, $n = 11$.

target concentrations increased/decreased because more/less hydrogen peroxide was generated on the bioactive channels and moved into the CL detection areas. In contrast, the presence of the bioactive channels without glucose and uric acid did not contribute to the signal and exhibited a very low background. The results indicated the great possibility of simultaneous quantitative analysis of glucose and uric acid on this μPCAD biosensor.

The performance of this μPCAD biosensor was verified by applying 30 μL of mixed samples of glucose and uric acid at various concentrations in TBS. Under the optimal conditions, as shown in Fig. 5B, the calibration graph was linear over the range of 0.42 to 50 mmol L^{-1} for glucose and the range of 1.4 to 47 mmol L^{-1} for uric acid. The linear regression equations for glucose and uric acid are shown in Fig. 5B. The relative standard deviation of this method was below 2.4% for glucose and 2.6% for uric acid in 11 repeated measurements. The detection limit was 0.14 mmol L^{-1} for glucose and 0.52 mmol L^{-1} for uric acid. The normal levels of glucose and uric acid in urine are 0.1–0.8 mmol L^{-1} and 1.5–4.4 mmol L^{-1} respectively.[54] Thus the concentration of glucose/uric acid in physiology levels that falls within the linear range of this method can be quantified by this μPCAD biosensor.

The reproducibility and storage stability of the μPCAD biosensor were also examined. The relative standard deviation (RSD) of the μPCAD biosensor response to 15.0 mmol L^{-1} glucose/uric acid in artificial urine[55] was 8.9% for 11 successive measurements. When the μPCAD biosensor was stored dry at 4 °C (sealed) and measured at intervals of 1 week, both the enzyme and the CL reagent maintain good performance after 10 weeks (data in the ESI†). On the other hand, the CL response to glucose/uric acid had no apparent decrease, indicating that the fabricated μPCAD was stable for storage or long-distance transport in developing countries.

To investigate the feasibility and reliability of this μPCAD biosensor for analysis of glucose and uric acid in complex biological samples, the assay was examined with different concentrations of glucose/uric acid in an artificial urine sample.[55] Except glucose and uric acid, the artificial urine solution contained 1.1 mmol L^{-1} lactic acid, 2.0 mmol L^{-1} citric acid, 25.0 mmol L^{-1} sodium bicarbonate, 170.0 mmol L^{-1} urea, 2.5 mmol L^{-1} calcium chloride, 90.0 mmol L^{-1} sodium chloride, 2.0 mmol L^{-1} magnesium sulfate, 10.0 mmol L^{-1} sodium sulfate, 7.0 mmol L^{-1} potassium dihydrogen phosphate, 7.0 mmol L^{-1} dipotassium hydrogen phosphate, and 25.0 mmol L^{-1} ammonium chloride all mixed in TBS. The pH of the solution was adjusted to 6.4 by addition of 1.0 M hydrochloric acid. The assay results indicated that the influence of lactic acid and citric acid in normal levels on the glucose and uric acid response were acceptable, namely 3–8% (detailed data in the ESI†). The other components in the artificial urine have no obvious influence on the glucose and uric acid response, indicating that this μPCAD biosensor is feasible for complex biological samples.

Conclusions

In this work, a μPCAD biosensor was developed, for the first time, that allows for simultaneous, fast, and convenient determination of glucose and uric acid. The combination of the μPAD and CL method makes the final products inexpensive, low-volume, portable, disposable, and easy-to-use. The samples are transported on the paper substrate by capillary action, making the use of an external pumping system unnecessary. In addition, the WCP#1 also acted as a filter paper to decrease the possible interference of coexisting substances to the CL reaction. This μPCAD biosensor was patterned using a cutting method, with which it is much easier to realize batch production than with other pattern methods. There is no requirement for expensive additional equipment, no transfer of the substrate between different process steps, and no need for curing of polymers or development of photoresists. The possible CL assay principle of this μPCAD biosensor was demonstrated. The simultaneous determination of glucose and uric acid could be achieved by differing the distances that the glucose/uric acid solutions traveled. This μPCAD biosensor could provide reproducible results upon storage at 4 °C for at least 10 weeks. Hence, we have employed the CL method on a μPAD for the simultaneous determination of glucose and uric acid for the first time. We believe this novel μPCAD biosensor has potential as a powerful tool for point-of-care diagnosis compared to traditional methods.

Acknowledgements

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