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A novel chemiluminescence paper microfluidic biosensor based on enzymatic reaction for uric acid determination

Jinghua Yu*, Shoumei Wang, Lei Ge, Shenguang Ge

School of Chemistry and Chemical Engineering, University of Jinan, Jinan 250022, PR China

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ABSTRACT

In this work, chemiluminescence (CL) method was combined with microfluidic paper-based analytical device (μPAD) to establish a novel CL μPAD biosensor for the first time. This novel CL μPAD biosensor was based on enzyme reaction which produced H_2O_2 while decomposing the substrate and the CL reaction between rhodanine derivative and generated H_2O_2 in acid medium. Microchannels in μPAD were fabricated by cutting method. And the possible CL assay principle of this CL μPAD biosensor was explained. Rhodanine derivative system was used to reach the purpose of high sensitivity and well-defined signal for this CL μPAD biosensor. And the optimum reaction conditions were investigated. The quantitative determination of uric acid could be achieved by this CL μPAD biosensor with accurate and satisfactory result. And this biosensor could provide good reproducible results upon storage at 4 °C for at least 10 weeks. The successful integration of μPAD and CL reaction made the final biosensor inexpensive, easy-to-use, low-volume, and portable for uric acid determination, which also greatly reduces the cost and increases the efficiency required for an analysis. We believe this simple, practical CL μPAD biosensor will be of interest for use in areas such as disease diagnosis.

1. Introduction

It has been estimated that 10–15% of the United States population will develop a kidney stone during their lifetime. Among the urinary components serving as the building blocks of kidney stones, uric acid, one of the final products of protein metabolism, is the most abundant molecular crystalline component. Uric acid is a very important biomolecule, which are the symptoms of several diseases such as gout, hyperuricaemia and hypertension and so on. Consequently, uric acid level as one of the important parameters monitored in urine and blood serum, simple methods that can be used conveniently for uric acid monitoring at home are required. Various methods have been reported for its determination, such as electrochemical methods (Uchiyama et al., 1990; Keedy and Vadgama, 1991; Gilmartin and Hart, 1994; Motonaka et al., 1994), fluorimetry (Galban et al., 2001), spectrofluorometric methods (Martinez-Pérez et al., 2003; Oda et al., 2004), colorimetric methods (Tivedi et al., 1978; Duncan et al., 1982; Kayamori and Yoshiaki, 1994) and so on. However, most of these methods have inherent problems in uric acid determination: expensive instruments, complex operations and trained personnels, which limit their applications.

Microfluidics, as a focus in analysis field, has been widely applied in many biological assays, such as electrophoresis (Manz et al., 1992), immunoassays (Jiang et al., 2003; Yang et al., 2008; Liu et al., 2009; Shi et al., 2007; Chen et al., 2009a), nucleic acid amplification analysis (Northrup et al., 1993; Schaerli et al., 2009; Huang et al., 2007), cell manipulations (Sun et al., 2009; Chen et al., 2008, 2009b; Goĭmez-Sjo¨berg et al., 2007) and so on, due to its advantages of working with little reagent and short reaction time. However, many types of materials used to fabricate microfluidic device, such as glass and polymer, are generally high cost, nondisposable and not easy to be patterned which limit their applications. Furthermore a significant portion of the world's population could never afford the cost of such advanced devices. To this end, the development of microfluidic devices has been spurred by the desire to produce point-of-care diagnostic devices, which are extremely low cost and require minimal external instrumentation for obtaining quantitative information (Altinier et al., 2001; Sia and Kricka, 2008; Whitesides, 2006).

Paper, a cellulose fiber web with high surface area, has the potential to be good alternative for traditional microfluidic device materials because it is relatively cheap, abundant, disposable, and easy to use, store and transport. The structure and porosity of paper are highly controllable, and the surface nature of paper-based materials is ready for modification. Therefore, paper has been already served as a medium for chemical analysis for many decades, even centuries (Carrilho et al., 2009b). Paper-based assays have been used for a variety of simple diagnostic tests as paper strips,

* Corresponding author. Tel.: +86 531 82767161.
E-mail addresses: wangshoumei1989@sina.com, jndxgelei@gmail.com, 306375767@qq.com, gl-198602@163.com (J. Yu).

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and can perform rapid analysis without any supporting equipments due to capillary action. However, the paper strip tests often suffer from the fact that they are only the "yes/no" detection, not quantitative, and lack the ability for multiplex analysis (Piia, 2005; Lim et al., 2005). More recently, paper has been patterned into microchannels (Martinez et al., 2007; Zhao and Berg, 2008; Abe et al., 2008; Li et al., 2008; Martinez et al., 2008a,b) using photolithography methods (Martinez et al., 2007, 2008a,b), plotting methods (Bruzewicz et al., 2008), plasma oxidation method (Li et al., 2008), cutting (Fenton et al., 2009), inkjet printing method (Abe et al., 2008), or wax printing methods (Carrilho et al., 2009a; Lu et al., 2009) to create microfluidic paper-based analytical device (μPAD), which combines the simplicity of paper strip tests and the complexity of the conventional lab-on-chip devices (Martinez et al., 2007; Zhao et al., 2008; Dungchai et al., 2009; Martinez et al., 2009; Bruzewicz et al., 2008). The μPAD is a class of microfluidic systems capable of performing truly disposable, inexpensive, and portably total analysis, especially microchannels patterned on paper can be used for sample pretreatment and separation. Therefore, we believe they will be well-suited for diagnostic applications in developing countries, in the field by first responders or in home healthcare settings.

Recently, there have been a few detection methods established on paper, such as colorimetric methods (Martinez et al., 2008a; Zhao et al., 2008; Dungchai et al., 2010) and electrochemical methods (Dungchai et al., 2009; Carvalhal et al., 2010; Apilux et al., 2010; Nie et al., 2010). Absorbance and fluorescence methods have been proved to be the potential detection methods on paper (Carrilho et al., 2009b). Chemiluminescence (CL) detection is one of the most popular detection methods (Zhang et al., 1999). And CL detection has become quite a useful method due to its high sensitivity and high compatibility with micromachining technologies. Therefore, for over 30 years, the development of CL has provided a well-established and widely applied branch of Analytical Chemistry (Su et al., 2007). However, to our best knowledge, no reports about establishing CL biosensor on μPAD have been published. In this paper, we demonstrate a novel rhodanine derivative CL system between rhodanine derivative and generated H_2O_2 in Tris–HCl buffer based on the long-term basic researched of rhodanine derivative CL system in our lab (Yu et al., 2009, 2010, 2011) and find that this CL system has a well-defined CL response on μPAD. In this work, a rhodanine derivative 3-p-nitrylphenyl-5-(4′-methyl-2′-sulfonophenylazo) rhodanine (M4NRASP) is used as a model rhodanine (Yu et al., 2009).

The purpose of this work is to develop a novel CL enzyme biosensor based on μPAD, which pursues the portable sensing, for the fast, simple detection of uric acid in artificial urine. The CL μPAD biosensor was based on facile enzyme/reagent immobilization and newly demonstrated cut-patterned technology (Fenton et al., 2009). This cut pattern, which is never exposed to photoresists or other polymers and inks that could contaminate it or interfere with the reaction on paper, will be more suitable to fabricate CL μPAD biosensor. The possible CL assay principle of this CL μPAD biosensor was explained. This CL μPAD biosensor possesses high sensitivity, good selectivity and requires small sample volume, short response time. Finally, this CL μPAD biosensor was applied to the determination of uric acid in artificial urine.

2. Materials and methods

2.1. Reagents and materials

All reagents were of analytical grade or above and used as received without further purification. Uric acid was purchased from Sigma–Aldrich (St. Louis, MO). Urate oxidase (UOx) (E.C.1.7.3.3 from arthrobacter globiformis; 18,000 U g^{-1}) was obtained from Sigma (Shanghai, China). 3-p-Nitrylphenyl-5-(4′-methyl-2′-sulfonophenylazo) rhodanine (M4NRASP) was obtained from our lab (Yu et al., 2009). Whatman chromatography paper #1 (200 mm × 200 mm) was obtained from GE Healthcare Worldwide (Pudong Shanghai, China) and used with further adjustment of size. This type of Whatman paper was chosen because of its uniform composition (relative to other types of paper) and lack of additives that affect flow rate and CL reaction. Oxygen-saturated Tris–HCl buffer solution (TBS) (pH = 6.8) is used in the experiment for enzyme-substrate reaction and CL determination.

2.2. Fabrication of the paper device

The schematic representation of the CL μPAD biosensor stacked between two layers of water-impermeable single-sided adhesive tapes was shown in Fig. 1. The CL μPAD biosensor was fabricated by stacking one layer of paper (patterned in ways that channel the flow of fluid within paper) between two layers of water-impermeable single-sided adhesive tapes. The top-layer of water-impermeable single-sided tape was patterned to form a square hole (3 mm side length) in it. The pattern of the hole was designed with Adobe Illustrator software (Adobe Systems, Inc.) and fabricated by a rectangle knife cutter. Cutting method was used to pattern Whatman filter paper #1 according to previously reported methods (Fenton et al., 2009). The middle paper layer was comprised of three independent components: sample injection area, bioactive channel and CL detection area. It was patterned using a computer controlled *X–Y* knife plotter through a three sequential overlapping cuts method to avoid the tearing of the paper. The first two of sequential cuts penetrate only part way through the chromatography paper. Following cutting operations, the removal of unwanted material generated from the pattern was performed manually. The size of each component was shown in Fig. 1. The bioactive channel was prepared by the immobilization of enzyme on the paper microchannel. The enzymes were immobilized by simple adsorption technique into the fiber structure of the paper. Immobilization was carried out in batch by immersing the paper microchannels (ten paper microhannels each time in this study) into concentrated enzyme solution at 4 °C for 5 min. After this period, enzyme immobilized papers were removed from the enzyme solution and dried in freeze-drying box. The CL detection area were prepared in batch by immersing rectangular papers (ten rectangular papers each time in this study) into rhodanine derivative ethanol solution for 5 min, and dried at room temperature in air. Briefly, the independent component of the middle paper layer were firstly attached and assembled onto the bottom-tape layer, and then the device was covered by the top-tap layer to seal the paper layer. The hole in the top-tap should be aligned to the sample injection area. These two steps can be finished within 10 min and will produce the cut-patterned paper device.

2.3. Chemiluminescence assay procedure of this CL μPAD biosensor

As shown in Fig. 1, CL emission was measured using a computerized ultraweak luminescence analyzer (Type RFL-200, manufactured at Xi'an Remex Analysis Instrument Co, Ltd, Xi'an, China). There is a newly designed device-holder at the bottom of the cassette to fix the position of the μPAD. And the cassette could be shut with a black metallic cover that has an injection hole for sample injection. When the μPAD was put into the holder, the sample injection area was aligned to the injection hole and the CL detection area was aligned to the photomultiplier of the analyzer. For CL detection, 20 μL of sample solution containing a desired concentration of uric acid in TBS was dropped onto the sample injection area through the hole by a pipette. The sample solution was migrated toward the CL detection area to obtain the CL signal. And the signal

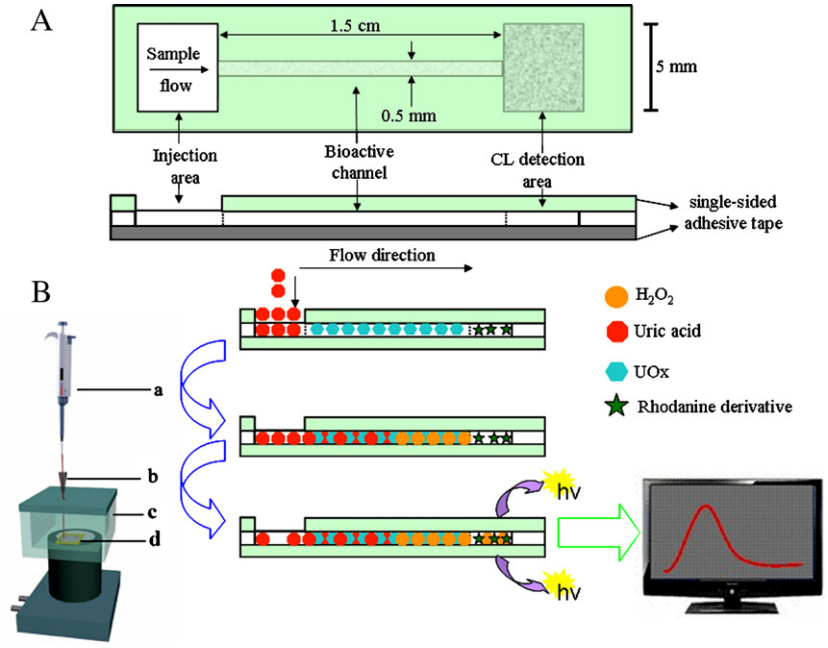

Fig. 1. Schematic diagram of uric acid determination showing its main components: top view and section view (A); schematic representation of the uric acid determined in microchannel (B): (a) pipette; (b) rubber seal cone; (c) cassette; (d) device-holder.

was recorded using a computer. Data acquisition and treatment were performed with RFL software. The concentration of sample was quantified by the peak height of the CL signal.

3. Results and discussion

3.1. Chemiluminescence assay principle of this CL μPAD biosensor

This CL μPAD biosensor, fabricated by cut paper and immobilized reagents, consists of three independent components: sample injection area, bioactive channel and CL detection area (Fig. 1A). All the components were assembled between two water-impermeable single-sided adhesive tapes. As shown in Fig. 1B, during the assay, 20 μL sample solution was dropped onto the sample injection area by a pipette. The sample flew toward the CL detection area without any supporting equipments due to capillary action and then reacted with the UOx on the bioactive channel according to the specific enzyme-substrate reaction. The generated H_2O_2 and TBS continued to migrate, governed by Lucas–Washburn equation (Mendez et al., 2009), along the bioactive channel. Then the H_2O_2 reached the CL detection area at the adherent point with a relatively high fluid velocity. Therefore, the reaction between H_2O_2 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. Then as the reaction proceeded, the CL intensity went down, due to the loss of generated H_2O_2 in the advancing liquid front. Quantitative analysis was realized by reading the peak height. The more uric acid in the sample, the more H_2O_2 would be migrated to the CL detection area, which leads to the increase of CL intensity. Consequently, according to the possible principle described above, the CL intensity on the CL detection area would be proportional to the concentration of uric acid in the sample.

The CL responses of this CL μPAD biosensor corresponding to different systems were shown in Fig. 2. As can be seen in Fig. 2, any one of rhodanine derivative, H_2O_2 or uric acid is not able to obtain robust CL intensity (curves 1, 2 and 3). When the UOx and uric acid were, respectively, added into the rhodanine derivative CL system, no CL intensity was obtained (curves 4 and 5). Once the uric acid solution flew through adsorbed enzyme molecules in bioactive channel, allantoic acid and H_2O_2 were produced. And the CL intensity increased remarkably when H_2O_2 contacted with rhodanine derivative and the CL intensity increased as the concentration of uric acid solution increasing (curves 6 and 7). Consequently, it was found that H_2O_2 was essential for CL generation. And the rhodanine derivative CL system was used to uric acid determination in this study. The results indicated the great possibility of quantitative analysis of uric acid on this CL μPAD biosensor.

3.2. Optimization of the CL μPAD biosensor system

The width of the bioactive channel is designed to decrease the reagent requirement and increase the fluid velocity as narrowly as possible according to the Darcy's Law (Mendez et al., 2009). Considering the actual resolution of the knife plotter and the property of the paper, 0.5 mm is selected as the final width of the bioactive channel. In this CL μPAD biosensor, the influence of pH value of TBS on the enzymatic reaction activity and the generated CL signal

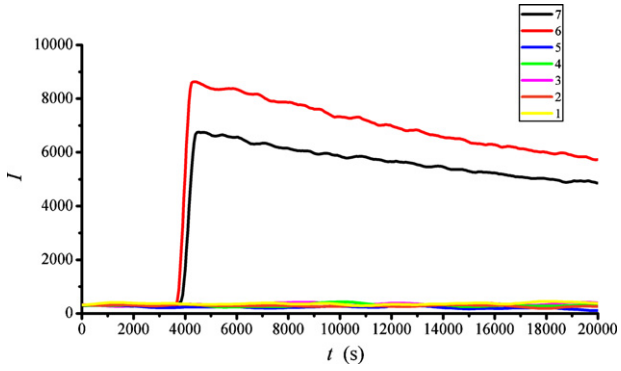

Fig. 2. The chemiluminescence intensity under different system: (1) rhodanine derivative solutions; (2) H_2O_2; (3) uric acid (15 mmol L^{-1})–TBS solutions; (4) rhodanine derivative solutions + uric acid (15 mmol L^{-1}) + H_2O; (5) rhodanine derivative solutions + uric acid (15 mmol L^{-1}); (6) rhodanine derivative solutions + UOx + uric acid (15 mmol L^{-1}); (7) rhodanine derivative solutions + UOx + uric acid (20 mmol L^{-1}).

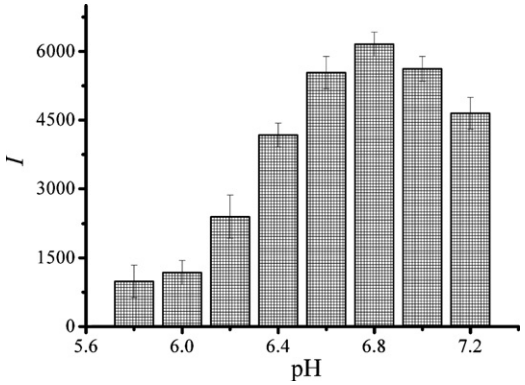

Fig. 3. Effect of the pH of TBS to the enzyme activity of UOx.

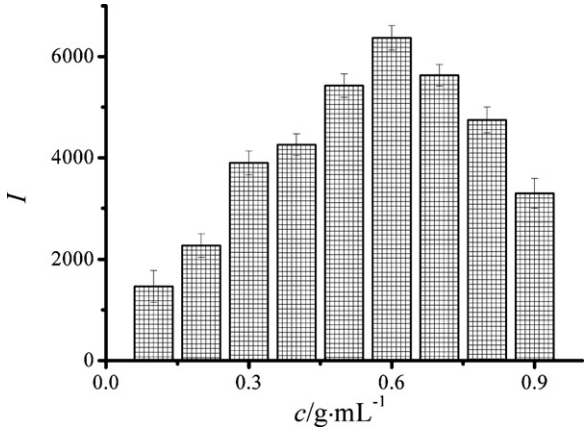

Fig. 5. Effect of the UOx amount.

is an important factor that determines the overall response of the biosensor. In view of the nature of traditional rhodanine derivative CL reaction, which is more favored under acid conditions (Yu et al., 2010, 2011), an acid medium would improve the sensitivity of the system. However, the optimal pH value for UOx is 7 (Suzuki et al., 2004). Therefore, the effect of TBS pH on the biosensor is investigated through changing the pH value of TBS. Using the ultraweak luminescence analyzer, the CL intensity reaches the highest value at pH 6.8, which is shown in Fig. 3. Therefore, this CL μPAD biosensor is performed at pH 6.8 throughout this study.

As the CL reagent, the amount of loaded M4NRASP on the CL detection area affects the CL response of this CL μPAD biosensor directly. To probe the optimal amount of M4NRASP for the assay, they are diluted into various concentrations and investigated the influence on CL intensity. It is found in Fig. 4 that 65.0 mmol L^{-1} is the optimal concentration to save the reagent. Therefore, 65.0 mmol L^{-1} M4NRASP is routinely used throughout the entire study. The amount of enzyme, loaded on the bioactive channel by physical absorption, also affects the CL sensitivity of this device. To probe the optimal amount of the absorbed enzyme for the assay, the influence of various concentrations of UOx on signal-to-noise (S/N) ratio of the CL μPAD biosensor for 15.0 mmol L^{-1} uric acid is investigated (Fig. 5). The S/N ratio is found to be highest for dispensing 0.6 g mL^{-1} UOx for 5 min. However, the decrease of S/N at a higher concentration is resulted from the increase of background signal due to too high of a concentration of enzyme while that at lower concentration is ascribed to the decrease of signal due to too low of the amount of enzyme. Therefore, 0.6 g mL^{-1} UOx is routinely used as the optimal concentration throughout the entire study. In addition, too high concentration of enzyme may block the porous structure of the paper and decrease the capillary action which decreased the efficiency and sensitivity of the biosensor. All the other device sizes and shapes are designed to decrease the reagent requirement as much as possible.

3.3. Reproducibility and storage stability of this CL μPAD biosensor

To be useful for practical applications, prepared CL μPAD biosensor must be able to retain their activity during storage. Therefore, the storage stability and reproducibility of the CL μPAD biosensor stored dry at 4 °C (sealed) was investigated by measuring the biosensor response at intervals of 1 week. After 10 weeks of testing, the response CL intensity diminished to 97.8% of the original response, and both the enzyme and the CL reagent still maintained good performance. The relative standard deviation (RSD) of the biosensor response to 15.0 mmol L^{-1} uric acid was 0.29% for 11 successive measurements. On the other hand, the response to uric acid had no apparent decrease, indicating that the fabricated CL μPAD biosensor was stable for storage or long-distance transport in developing country.

3.4. Analytical performance of this biosensor for uric acid measurement

The performance of this CL μPAD biosensor was verified by applying 20 μL sample solution of uric acid at various concentrations in TBS. Under the optimal conditions, the quantitative calibration graph was linear over the range of 2.6–49.0 mmol L^{-1}, as shown in Fig. 6, the linear regression equation was $\Delta I = 692.22 + 383.61c$

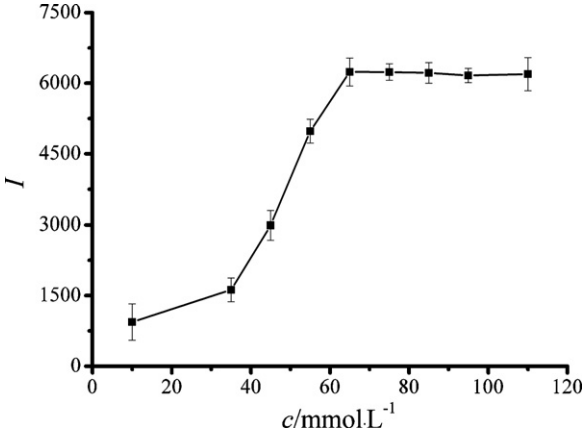

Fig. 4. Effect of the rhodanine derivative concentration.

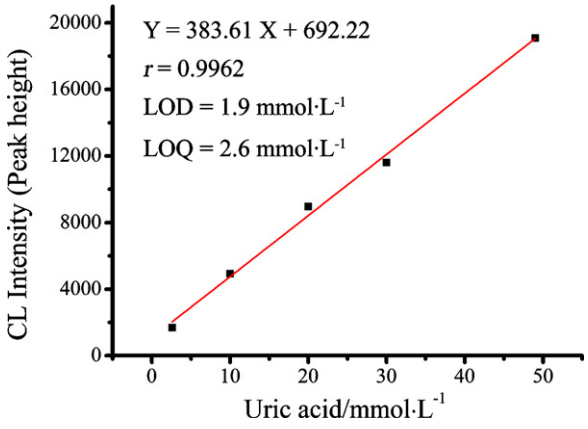

Fig. 6. Calibration line of this CL μPAD biosensor.

(c: mmol L^{-1}) (r = 0.9962) (where ΔI was the relative CL intensity (peak height) and c was the concentration of uric acid). The relative standard deviation of this method was below 2.8% in 11 repeated measurements. The limit of detection (LOD) and limit of quantification (LOQ) were 1.9 mmol L^{-1} and 2.6 mmol L^{-1}, respectively. The normal levels of uric acid in urine are 1.5–4.4 mmol L^{-1} (Tietz, 1995). Therefore, the concentration of the uric acid in physiology level that falls within the linear range can be detected.

To investigate the feasibility of this CL μPAD biosensor system for analysis of uric acid in complex biological sample, the assay was performed with different concentrations of uric acid in the artificial urine sample (Ellerbee et al., 2009). Except uric acid, the artificial urine solution contained 1.1 mmol L^{-1} lactic acid, 2.5 mmol L^{-1} calcium chloride, 90 mmol L^{-1} sodium chloride, 2.0 mmol L^{-1} magnesium sulfate, 10 mmol L^{-1} sodium sulfate, 2.0 mmol L^{-1} citric acid, 25 mmol L^{-1} sodium bicarbonate, 170 mmol L^{-1} urea, 7.0 mmol L^{-1} potassium dihydrogen phosphate, 7.0 mmol L^{-1} dipotassium hydrogen phosphate, and 25 mmol L^{-1} ammonium chloride all mixed in TBS. The pH of the solution was adjusted to 6.8 by addition of 1.0 M hydrochloric acid. The recovery experiments were conducted by spiking the artificial urine with uric acid at different concentrations, recoveries of uric acid were obtained in the range of 97–104%. The assay result indicated that the influence of lactic acid, citric acid in normal level on the uric acid response were acceptable, namely 4–7%. The other components in the artificial urine had no obviously influence on the uric acid response, indicating that this CL μPAD biosensor system was feasible in the complex biological sample.

4. Conclusions

We demonstrate here for the first time the coupling of CL method and μPAD to provide portable, simple, rapid quantitative measurement of critical health marker-uric acid in artificial urine. In this work, a novel CL enzyme biosensor based on μPAD was developed for uric acid determination, which combines sensitive enzyme receptor with rhodanine derivative CL system. The capillary action pushes the samples forwards on the paper substrate, which makes the use of an external pumping system unnecessary. In addition, the paper could also act as a filter to decrease the possible interference of coexisting substances to the CL reaction.

This CL μPAD biosensor is fabricated using cutting method. Therefore, there are no requirement for expensive additional equipments, no transfer of the substrate between different process steps, and no need for curing of polymers or development of photoresists. On the other hand, unlike the colorimetric and electrochemical μPAD biosensor, the enzyme receptor and chemiluminescence detector on CL μPAD biosensor are separated and independent, this could remarkably maintain the activity and enhance the stability of enzyme. The reagent type and amount required on CL μPAD biosensor are much less than the colorimetric and electrochemical μPAD biosensor. This could obviously decrease the reagent quantity demanded and simplify the fabrication procedure to realized industrially batch production. In addition, this CL μPAD biosensor is sealed between two layers of water-impermeable single-sided adhesive tapes, this could further eliminate the evaporation of the sample solution and decrease influence of the external interference such as wet air and dust.

The possible CL assay principle of this CL μPAD biosensor is explained. The optimal conditions of microchannel have been studied. It is of great importance for widening the application field of paper device as experimental carrier for CL biosensor. This CL μPAD biosensor was satisfactory when applied to the determination of uric acid in artificial urine. This CL μPAD biosensor could provide reproducible results upon storage at 4 °C for at least 10 weeks and good reproducibility. The relatively low detection limit, wide detection range, simple determination procedure and high stability make this CL paper microfluidic biosensor a powerful and potential tool for uric acid diagnosis.

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