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ORIGINAL RESEARCH

Effect of the geometry of microfabricated flow reactors on chemiluminescent detection of epinephrine with lucigenin

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ABSTRACT: Three types of flow reactors with different lengths (12–126 mm) and widths (0.4–2.0 mm) of channel were made on the silicon chip by microfabrication techniques for the chemiluminescent (CL) detection of epinephrine (EP) with lucigenin (Luc). The volume of each CL reactor was about 10 μL. A solution containing EP and Luc and a solution containing NaOH and periodate were injected successively into each inlet of the CL reactor in the range 20–100 μL/min with a pressure-driven flow system. The intensity of light emission was dependent on the geometry of the flow reactors. These results could be explained in terms of the differences in the diffusion length of the reactants in the flow reactors. The maximum light emission were linearly correlated, with the concentrations of EP over the range from the detection limit of 5.0×10^{-8} mol/L up to 5.0×10^{-6} mol/L on the use of the CL reactor with the most promising geometry. Copyright © 2001 John Wiley & Sons, Ltd.

KEYWORDS: chemiluminescence; lucigenin; epinephrine; microanalysis system; FIA

INTRODUCTION

Microscale total analysis systems (μTAS) have been developed for performing analytical procedures (1–3). Microfabricated devices for μTAS are designed with a channel network etched on a quartz glass chip or a silicon chip using standard photolithography, wet chemical etching and bonding techniques. An advantage of μTAS, relative to analytical systems of conventional size, is volumetric reduction of samples and analytical reagents. However, the sensitivity of a detection method decreases with a decrease of the amount of analyte in the fluid. Therefore, sensitive detection systems are required for μTAS. Fluorescence methods have been commonly applied to capillary electrophoresis integrated on a microchip. A laser is used as a light source in fluorescence methods, since a laser is suitable for being focused on the microfabricated channel.

On the other hand, a chemiluminescence (CL) method is widely used for a sensitive detection method. In addition, the instrument for the CL method is relatively simple compared with that for the spectrophotometric method, since a light source is not required in a CL detector. Therefore, a CL method has been recently applied to detection in μTAS. The luminol CL catalysed by horseradish peroxidase with peroxide was investigated in a post-separation detection of mouse IgG by microchip-based capillary electrophoresis (4). Wu *et al.* applied the luminol and peroxyoxalate CL methods to the detection of H_2O_2 in the integrated flow injection analysis (FIA) system with gravity as a driving force (5). The peroxyoxalate CL reaction was also applied to the detection of dansyl amino acids separated in microchip capillary electrophoresis (6). A micromachined mixer/reactor has been developed for the luminol CL detection of Cr(III) in a continuous flow injection mode (7). In addition, scaling theory has been described for explaining the dependence of the diffusion length on the CL signal strength (7).

In this study, we fabricated three types of flow reactors with different channel patterns by considering the diffusion length of the reactants in the flow reactors. The CL reaction of 10, 10′-dimethyl-9, 9′-biacridinium dinitrate (lucigenin) with epinephrine (EP) (8–10) was observed in a continuous flow injection mode. The factors affecting the intensity of light emission were characterized in terms of channel patterns, flow rate and reagent concentrations. The progress of the CL reaction in the flow reactors was also imaged by a cooled charge-coupled device camera.

MATERIALS AND METHODS

Reagents

Lucigenin (Luc) was obtained from Tokyo Kasei (Tokyo,

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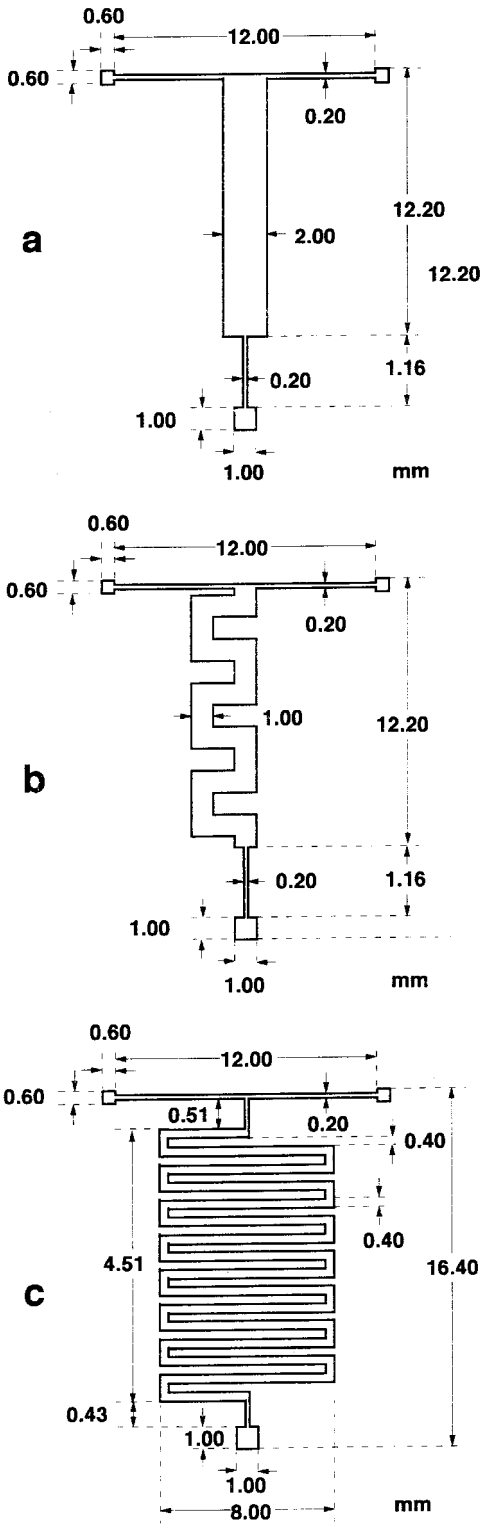

Figure 1. Three types of flow reactors: (a) channel pattern 1; (b) channel pattern 2; (c) channel pattern 3.

Japan). Epinephrine (EP) and periodate sodium were bought from Wako Pure Chemical Industries (Tokyo, Japan). An EP solution was prepared daily and was acidified to pH 2 with HCl. The base solutions were prepared from sodium hydroxide. All chemicals used were guaranteed-grade reagents and were used without further purification. All solutions were prepared with ultrapure deionized water from a Millipore Milli-Q water purification system. All of the reagent concentrations reported and shown in the figure captions are the initial solution concentrations.

Microfabrication of flow reactors on the silicon chip

Three-types of flow reactors were designed with different dimensions of 12 (L) × 2.0 (W) × 0.45 (D) mm (channel pattern 1), 24 (L) × 1.0 (W) × 0.45 (D) mm (channel pattern 2), and 126 (L) × 0.40 (W) × 0.28 (D) mm (channel pattern 3), as shown in Fig. 1. The volume of the flow reactors was 7–8 μL, which was about one-tenth of that in a flow cell using a conventional FIA system.

A silicon wafer [p-type (100) surface, 0.625 mm thick, optical polished] was washed sequentially with a mixture of water, ammonia water and hydrogen peroxide (5:1:1) at 80°C for 10 min, and a mixture of water, hydrochloric acid and hydrogen peroxide (6:1:1) at 80°C for 10 min. The wafer was thermally oxidized with steam under a nitrogen stream at 1100°C for 10 h in an oven. The thickness of the oxidized layer at this stage was approximately 2 μm. Spin-coating with positive-type photoresist (OFPR-800, Tokyo Ohka Kogyo, Japan) was carried out using a spin coater (Model 1H-DXII, Mikasa, Japan). After prebaking at 80°C for 15 min, the photoresist layer was photolithographically developed with a photomask to make the channel. Postbaking was performed at 130°C for 5 min. The oxidized layer was etched in a hydrogen fluoride:ammonium fluoride (1:7) buffer solution. The photoresist was then removed completely by washing with acetone and water. Anisotropic etching was carried out in a mixture of potassium hydroxide, isopropyl alcohol and water (100 g: 50 ml: 300 ml) at 75°C to make a channel on the silicon chip. The reversed side was also etched in the same manner with a photomask to make holes for the inlet and outlet. Next, the oxidized layer of the wafer was stripped with the buffered solution. Finally, the silicon wafer was anodically bonded to a Pyrex glass plate (Pyrex 7740, Corning). Anodic bonding was carried out at 400°C and 600 V for 3 h.

The volume of each flow reactor was calculated by measuring the width and depth of the channel, using a profile micrometer (Model VF-7510, Keyence, Japan) and a surface profiler (Model surfcom 300B, Tokyo Seimitsu, Japan), respectively. The volumes of channel patterns 1, 2 and 3 thus calculated were 8.4, 7.3 and 7.1 μL, respectively.

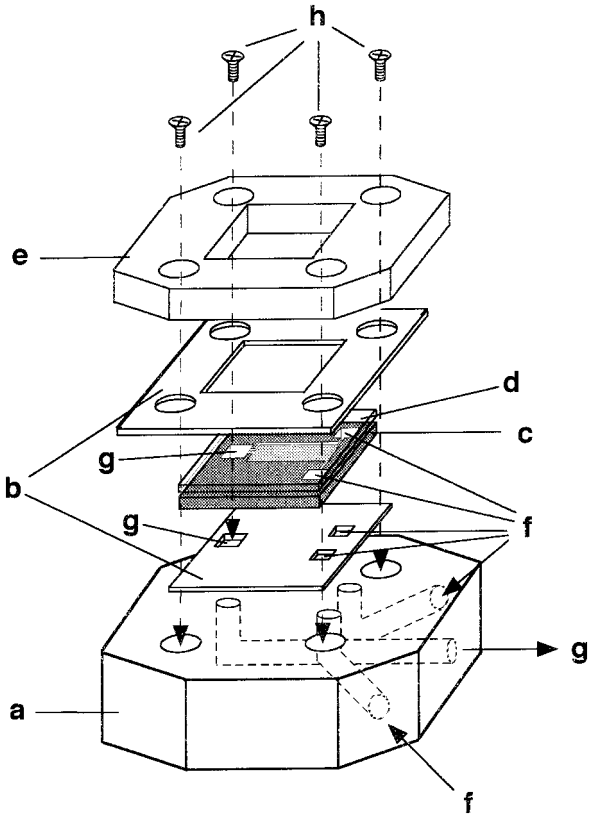

Figure 2. Structure of a chip-holder: (a) acrylate resin; (b) silicone rubber; (c) flow reactor; (d) Pyrex glass; (e) copper plate; (f) inlet hole; (g) outlet hole; (h) stainless screw.

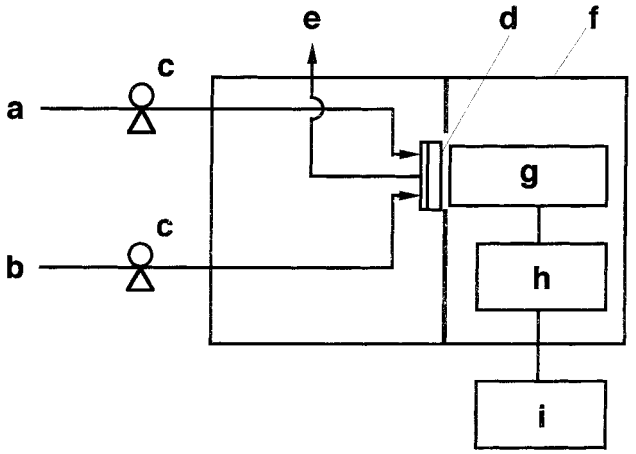

Figure 3. Continuous flow injection system for the CL detection of EP with Luc: (a) a solution containing NaOH and $NaIO_4$; (b) a solution containing EP and Luc; (c) a HPLC pump; (d) chip-holder; (e) waste; (f) CL detector; (g) photomultiplier; (h) amplifier; (i) recorder.

CL detection system of EP with Luc in the continuous flow injection mode

The flow reactor consists of an anisotropically etched silicon chip and a Pyrex glass plate. Each plate was a 25 mm × 25 mm square plate. The silicon chip has a channel, and penetrated holes at each end of the channel. The flow reactor was fixed on a chip-holder, as shown in Fig. 2. The chip-holder was faced to a photomultiplier tube (PMT) in a CL detector.

Fig. 3 shows a continuous flow injection diagram for the CL detection of EP with Luc. The flow system consisted of a HPLC pump (minimum flow rate, 5 μL/min; Model PU611, GL Sciences, Tokyo, Japan), a CL detector (Model ICA-3070, TOA Electronics Ltd, Tokyo, Japan) and a recorder (Model R-61, Rikadenki Kogyo, Tokyo, Japan). A 30 cm length of Teflon tubing (0.25 mm i.d.) connected the pump to the inlet of the chip-holder. A 0.01 mol/L solution of NaOH containing 5.0×10^{-4} mol/L periodate and a 1.0×10^{-4} mol/L solution (pH 4.2) of Luc containing EP were successively loaded from the T-shaped injection into the flow reactor. Light emission was detected by the PMT in the CL detector. The resultant photocurrent was converted to a voltage, whose value was displayed on the chart recorder. Light emission from the flow reactor was imaged by a cooled charge-coupled device (CCD) camera (Model C4880-30-24A, Hamamatsu Photonics, Hamamatsu City, and Japan). The CCD was cooled to −50°C to minimize the dark current.

RESULTS AND DISCUSSION

Light emission–time profiles observed in different flow reactors

The determination of EP, a group of catecholamines, is primarily useful for the diagnosis for various diseases and evaluation of their medical treatments. Therefore, many analytical methods have been developed for the determination of EP (11–13). We have previously shown that Luc reacts with EP to form 3-methylacridone and EP oxidants, with concomitant CL (10):

$$\text{Luc} + \text{EP} \longrightarrow \text{3-methylacridone} + \text{EP oxidants} + \text{light}$$

The CL reaction proceeds rapidly after an addition of a solution containing Luc and EP into an alkaline solution, and then the maximum of light emission is reached in a few seconds. The intensity of the maximum light emission is linearly related to EP concentration. Based on this finding, we proposed the application of the Luc CL method for the determination of EP. In addition, the oxidation rate of EP increased in the presence of periodate, thus resulting in the enhancement of the light emission (9). The detection limit for EP in the presence of

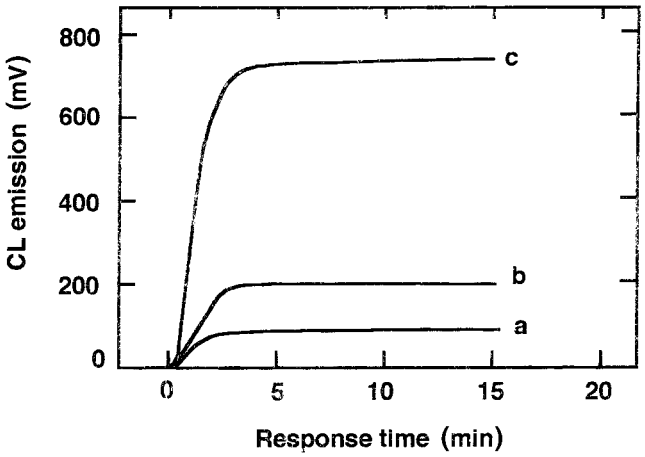

Figure 4. Typical CL response curves observed in the CL reaction of EP with Luc: (a) channel pattern 1; (b) channel pattern 2; (c) channel pattern 3.

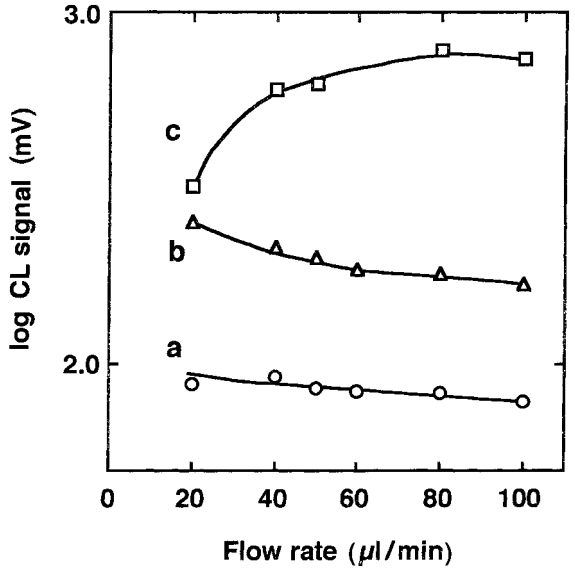

Figure 5. Effect of flow rate on the CL signal: (a) channel pattern 1; (b) channel pattern 2; (c) channel pattern 3. (1) Inlet of a solution containing 0.01 mol/L NaOH and 5.0×10^{-4} mol/L $NaIO_4$; (2) inlet of a solution containing 1.0×10^{-5} mol/L EP and 1.0×10^{-4} mol/L Luc.

periodate was 1.0×10^{-8} mol/L in the conventional batch method (9).

A two flow-line system was assembled as shown in Fig. 3 by taking into account the CL reaction of Luc with EP. A solution containing 1.0×10^{-4} mol/L Luc and 1.0×10^{-5} mol/L EP was prepared by dissolving the salts in 1.0×10^{-2} mol/L HCl, since no light emission is observed in the acidic condition. Periodate was added to a 0.01 mol/L solution of NaOH. The final pH was 11.6 after mixing the reagent solutions. The continuous flow injection mode was used for measuring the light emission in a steady state of the concentration of the reactants in the flow reactors.

Light emission was observed at a flow rate of 50 μL/min in each flow reactor. Typical CL response curves are shown in Fig. 4. The intensity of CL emission was constant after 3 min from the start of light emission in every channel pattern. A maximum CL emission is referred as a CL signal. The rate of light emission and the CL signal increased in the following order: channel pattern $3 > 2 > 1$. When the distance between the centres of the flows is defined as a diffusion length, the diffusion length decreases followed the channel pattern order: $1 > 2 > 3$. Therefore, the result as shown in Fig. 4 suggests that the efficiency of the CL reaction was dependent on the diffusion length of the reactants in the flow reactor. On the other hand, it takes 3 min to reach a CL signal plateau at a flow rate of 50 μL/min, although the volume of the CL reactor was about 10 μL. This result indicates that a long time is needed for the replacement of water in the flow reactor with the reactants.

Effect of flow rate on CL signal

The dependence of the CL signal upon flow rate was investigated in the range 20–100 μL/min. Fig. 5 shows the CL signal—the flow rate profiles. The CL signal decreased faintly with increasing flow rate in channel patterns 1 and 2. On the other hand, the CL signal increased gradually with an increase in the flow rate in channel pattern 3.

A fluid in a microfabricated channel is known to flow with laminar flow at a low flow rate (14). The solution containing NaOH and periodate was able to flow with laminar flow and no convective mixing across the two streams was possible. Therefore, it could be better to have a longer residence time for the diffusion of the reactants. Increase in the flow rate is responsible for a decrease in the residence time, thus resulting in the decrease of the CL signal in channel patterns 1 and 2. However, the CL signal increased with an increase in the flow rate in channel pattern 3. This result suggests that a distortion of laminar flow could occur in channel pattern 3, with an increase in the flow rate. However, the reason for the increase in the CL signal in channel pattern 3 is still not clear.

The CL signal observed contains the light emission of a blank, which was observed on the use of a solution containing no EP. We then measured the blank light emission according to the procedure, in which a solution containing 1.0×10^{-4} mol/L Luc alone was used in place of a Luc solution containing EP. The maximum of light emission in the absence of EP was referred as a blank CL intensity. These blank CL intensity-corrected CL values are referred to as a CL intensity.

The ratio of the CL intensity to the blank CL intensity (S:B ratio) was determined in each flow rate. The values of S:B ratio in each flow reactor are shown in Fig. 6. The

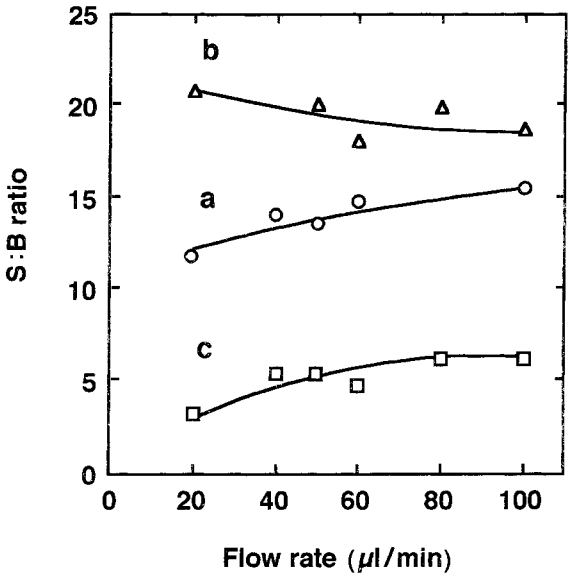

Figure 6. Effect of flow rate on CL signal:blank ratio: (a) channel pattern 1; (b) channel pattern 2; (c) channel pattern 3. (1) Inlet of a solution containing 0.01 mol/L NaOH and 5.0×10^{-4} mol/L $NaIO_4$; (2) inlet of a solution containing 1.0×10^{-5} mol/L EP and 1.0×10^{-4} mol/L Luc.

S:B ratio in channel pattern 2 was greater than that in channel patterns 1 and 3 in any flow rate. The decrease in the S:B ratio in channel pattern 3 was due to the remarkable increase in the blank CL intensity. For example, the signal CL intensities of channel patterns 1, 2 and 3 were 86, 201 and 627 mV at 50 μL/min, respectively. On the other hand, the blank CL intensities of channel patterns 1, 2 and 3 were 7.1, 10.6 and 151 mV, respectively. As a result, the most promising channel pattern with respect to the S:B ratio was channel pattern 2. In addition, the S:B ratio was almost constant in the flow range examined. Thus, the optimum flow range was chosen to be 50 μL/min.

Imaging of light emitted from the flow reactors

In order to confirm the progress of the CL reaction in the flow reactors, we then examined the imaging of light emitted from the flow reactors using a CCD camera. Fig. 7 shows the imaging of light emission at a flow rate of 50 μL/min. The site at which light emission was observed is shown in white in Fig. 7. In channel pattern 1, the light emission was mainly observed on the right side of the flow reactor, in which the solution containing EP and Luc flowed. In channel pattern 2, the light emission increased in the corner of the right side in the flow reactor. These results indicate that the diffusion of hydroxyl ions and periodate into the stream containing Luc and EP is more effective for the Luc CL reaction than that of Luc and EP into the stream containing hydroxyl ion and periodate. On

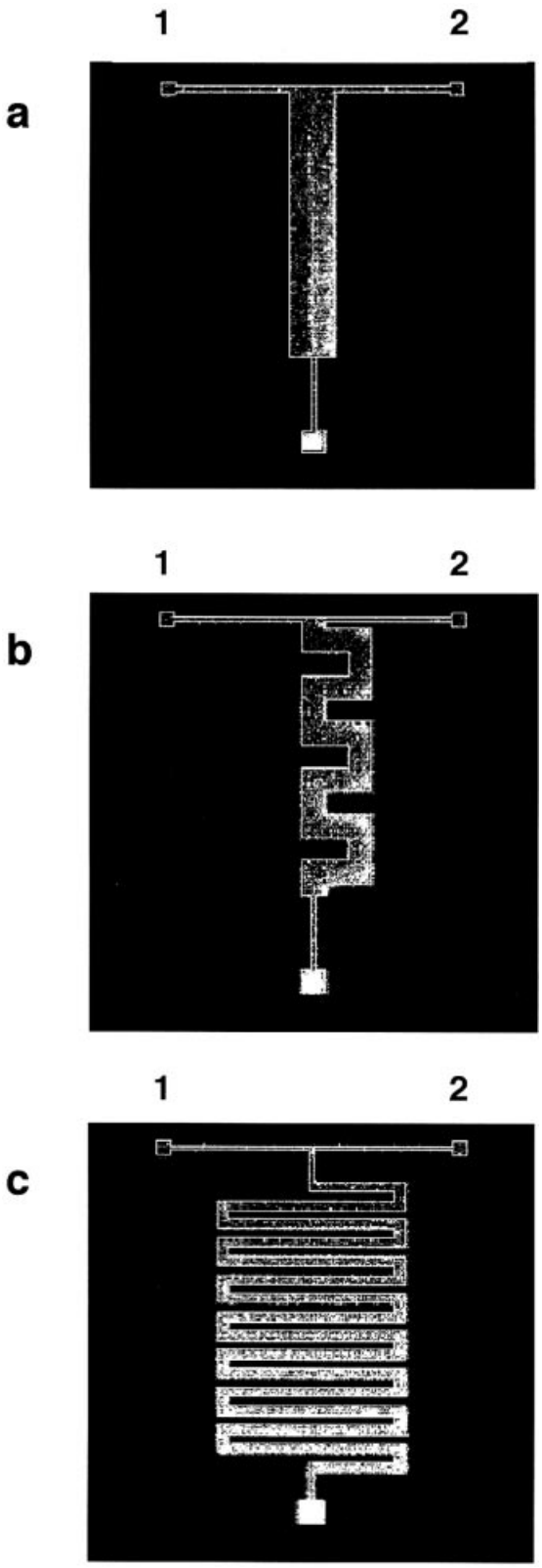

Figure 7. CCD camera imaging of light emission from the flow reactors: (a) channel pattern 1; (b) channel pattern 2; (c) channel pattern 3. (1) Inlet of a solution containing 0.01 mol/L NaOH and 5.0×10^{-4} mol/L $NaIO_4$; (2) inlet of a solution containing 1.0×10^{-5} mol/L EP and 1.0×10^{-4} mol/L Luc. The sites of light emission were shown in white.

the other hand, the light emission appeared from the whole area of the flow reactor in channel pattern 3. This result suggests that the distortion of laminar flow could occur in channel pattern 3. Therefore, the CL signal in channel pattern 3 could be increased with an increase in the flow rate, as shown in Fig. 5. As can be seen in Fig. 7, the area observed the light emission in the flow reactor increased in the following order: channel pattern $3 > 2 > 1$. This order was consistent with that of the rate of light emission and the CL signal. Therefore, when the reactor volume is constant, a geometry with a long and a narrow channel could be adequate for the flow reactor, taking into account the diffusion efficiency of the reagents between two streams.

Analytical results and parameters

The optimum concentrations of NaOH and periodate required to maximize the S:B ratio were determined using the flow reactor of channel pattern 2. The CL measurements were carried out using a solution containing 1.0×10^{-5} mol/L EP and 1.0×10^{-4} mol/L Luc at a flow rate of 50 μL/min.

The dependence of the S:B ratio upon the NaOH concentration was examined in the range of 0.01–0.1 mol/L. The S:B ratio was almost constant in the range of NaOH concentrations examined. Thus, the optimum concentration of NaOH was chosen to be 0.01 mol/L. Next, the influence of periodate concentrations on the S:B ratio was investigated in the range 5.0×10^{-5}–1.0×10^{-3} mol/L. The S:B ratio exhibited a broad maximum at 5.0×10^{-4} mol/L periodate. The optimum concentration of periodate was thus determined to be 5.0×10^{-4} mol/L.

The effect of periodate concentration on CL intensity appeared markedly in the CL reaction of EP with Luc by the batch method (10). The presence of periodate enhanced the CL intensity by factors of about 10 when compared with that in water alone in the batch method (10). On the other hand, in the continuous flow injection method using a microfabricated flow cell, the CL intensity increased by a factor of about three compared with that in water alone. Therefore, CL enhancement using periodate occurs more effectively in the batch method because the reactants are sufficiently mixed during the CL reaction in this method.

The calibration curve for EP on the use of channel pattern 2 was prepared under the optimum conditions thus established. The logarithmic calibration curve was linear over the range from the detection limit of 5.0×10^{-8} mol/L up to 5.0×10^{-6} mol/L with a slope of 0.32 and a correlation coefficient (R^2) of 0.972. The detection limit for EP was defined as the concentration of EP yielding an analytical signal equal to three times the standard deviation of the blank CL intensity. The relative standard deviation of the CL intensity for five successive experiments was 6.1% at 5.0×10^{-7} mol/L EP. The sensitivity for EP on the use of channel pattern 2 can be applied to the determination of EP in urine.

In conclusion, the intensity of light emission in the CL reaction of EP with Luc was dependent on the geometry of the flow reactors. The geometry with a long, narrow channel could be adequate for the flow reactor by accounting for diffusion efficiency of the reagents between two streams. Therefore, the investigation of the geometry of the flow reactor in which the reactants are more miscible could be important for the improvement of sensitivity in the continuous flow injection method.

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REFERENCES

1. Freemantle M. Downsizing chemistry. *C & EG* 1999; **February 22**: 27–36.
2. Figeys D and Pinto D. Lab-on-chip: a revolution in biological and medical sciences. *Anal. Chem.* 2000; **72**: 330–335A.
3. Lindner E and Buck RP. Microfabricated potentiometric electrodes and their *in vivo* application. *Anal. Chem.* 2000; 336–345A.
4. Mangru SD and Harrison DJ. Chemiluminescence detection in integrated post-separation reactors for microchip-based capillary electrophoresis and affinity electrophoresis. *Electrophoresis* 1998; **19**: 2301–2307.
5. Wu X-Z, Suzuki M, Sawada T and Kitamori T. Chemiluminescence on a microchip. *Anal. Sci.* 2000; **16**: 321–323.
6. Hashimoto M, Tsukagoshi K, Nakajima R, Kondou K and Arai A. Microchip capillary electrophoresis using on-line chemiluminescence detection. *J. Chromatogr. A* 2000; **867**: 271–279.
7. Xu Y, Bessoth FG, Eijkel CT and Manz A. On-line monitoring of chromium(III) using a fast micromachined mixer/reactor and chemiluminescence detection. *Analyst* 2000; **125**: 677–683.
8. Kamidate T, Yoshida K, Kaneyasu T, Segawa T and Watanabe H. Micelle-enhanced lucigenin chemiluminescence for the determination of catecholamines. *Anal. Sci.* 1990; **6**: 645–649.
9. Kamidate T, Kaneyasu T, Segawa T and Watanabe H. Enhancement of lucigenin chemiluminescence by peroxidate oxidation of adrenaline in cationic surfactants. *Chem. Lett.* 1991; **10**: 1719–1722.
10. Kamidate T, Ichihashi H, Segawa T and Watanabe H. Enhanced chemiluminescence of lucigenin with epinephrine in cationic surfactant micelles containing peroxidate. *J. Biolumin. Chemilumin.* 1995; **10**: 55–61.
11. Nozaki R, Kawamoto H and Moriyama H. Total free catecholamines assay by identification of its two functional groups and micro-flow injection chemiluminescence. *Luminescence* 1999; **14**: 369–374.
12. Zhu R and Kok WT. Determination of catecholamines and related compounds by capillary electrophoresis with postcolumn terbium complextion and sensitized luminescence detection. *Anal. Chem.* 1997; **69**: 4010–4016.
13. Arakawa H, Kanemitsu M and Maeda M. Chemiluminescent assay for catecholamines by the generation of hydrogen peroxide in basic solution and the use of isoluminol-microperoxidase. *Anal. Sci.* 1999; **15**: 1269–1272.
14. Kamholz AE, Weigl BH, Finlayson BA and Yager P. Quantitative analysis of molecular interaction in a microfluidic channel: the T-sensor. *Anal. Chem.* 1999; **71**: 5340–5347.

