Passive micromixer for luminol-peroxide chemiluminescence detection

Khoi Seng Lok, a Yien Chian Kwok b and Nam-Trung Nguyen a,b

Received 5th April 2011, Accepted 12th April 2011
DOI: 10.1039/c1an15280g

This paper reports a microchip with an integrated passive micromixer based on chaotic advection. The micromixer with staggered herringbone structures was used for luminol-peroxide chemiluminescence detection. The micromixer was examined to assess its suitability for chemiluminescence reaction. The relationship between the flow rate and the location of maximum chemiluminescence intensity was investigated. The light intensity was detected using an optical fiber attached along the mixing channel and a photon detector. A linear correlation between chemiluminescence intensity and the concentration of cobalt(II) ions or hydrogen peroxide was observed. This microchip has a potential application in environmental monitoring for industries involved in heavy metals and in medical diagnostics.

Introduction

Micromixers can be integrated in Lab-on-Chip (LoC) applications to improve mixing in laminar flow regime. Improved mixing can be achieved actively with external disturbance or passively with chaotic advection at high flow rate. A high flow rate also results in a shorter residence time and a faster analytical time. As mentioned above, micromixers are categorized as active and passive types. 3–8 Passive micromixers rely on modifications in the shape or the geometry of the mixing channel. Due to the lack of active components, passive mixers are relatively easy to implement in a LoC system. Most passive micromixers use chaotic advection to improve mixing. 9,10 Stroock et al. introduced the staggered herringbone mixer (SHM) based on chaotic advection. 11 Following this work, the channel designs were modified in various manners to improve mixing performance. 7,10 Mei et al. showed that having the herringbone pattern in the mixing channel improved the sensitivity of luciferase assay by three times. 11

Simple Y-shaped or T-shaped micromixers were often used in LoC application. Wei et al. reported a simple mixer for the determination of benzoyl peroxide in flour. 12 He et al. developed a LoC for micro-flow injection analysis (μFLA) to determine the amount of nitrite in food. 13 Marle and Greenway used a LoC to determine the amount of hydrogen peroxide in rainwater. 14 These devices have a simple serpentine microchannel as a mixing channel. Since simple T-mixers rely on molecular diffusion for mixing, the flow rate is limited to about 20 or 50 μl min⁻¹ and cannot be increased further. Based on Einstein–Smoluchowski diffusion theory, the time estimated for a protein with a diffusion coefficient of \( D = 10^{-6} \text{ cm}^2 \text{ s}^{-1} \) to move across half of a channel with a width of 1 mm is about 125 seconds. To warrant a residence time less than 125 seconds, a low flow rate is required for the operation of these devices.

Micromixers often serve as a microreactor. Tan et al. used a modified SHM for detection of nerve agent sarin in blood. 15 The modified SHM had been used as a reactor for nerve gas regeneration. Potassium fluoride was mixed with sarin-spiked whole blood to regenerate sarin for further detection. The optimized flow rate for this reactor was 20 μl min⁻¹, because the required time for enzymatic reaction was considered. Xu and Fang also used a SHM for glucose detection. 16 Although, the SHM was included to assist mixing, a low flow rate was used. Luminol and hexacyanoferrate were injected at 10 μl min⁻¹ into a 32 mm long SHM. Subsequently, glucose was injected at 25 μl min⁻¹ into a 35 mm long glucose oxidase reactor, composed of immobilized particle bed. The reactants were mixed in another 34.7 mm long SHM. When the sample flow rate increased from 10 to 70 μl min⁻¹, the detected signal decreased, apparently due to insufficient reaction time as claimed by the author. According to Lin’s work, 10 a SHM requires a mixing length of 60 mm to achieve a mixing efficiency of 95%. Hence, the length of a SHM warrants the sufficient residence time and proper mixing at higher flow rates. Using an appropriate micromixer as a microreactor, a LoC may facilitate fast mixing and high reaction efficiency at high flow rates. A good mixer allows adjusting the flow rate for the right residence time required by the enzymatic reaction time of the respective chemical system.

Chemiluminescence (CL) is a promising detection technique for LoC applications. This method does not require an excitation light source and gives lower background interferences. The only drawback of CL is that the light intensity diminishes rapidly when the reactants are mixed. Thus, a detector must be readily available in situ to capture the light signal once the reaction is
initiated. Many CL systems such as luminol, acridinium compounds, coelenterazine, dioxetanes and luciferase were used in applications such as immunoassays, receptor assays, DNA probes and biosensing. Luminal (5-aminophthalhydrazide) CL is a well-characterized reaction system that has been used for various applications such as monitoring of metals and other pollutants in water, immunoassays and DNA analysis and dating of human remains. During the reaction, luminal is oxidized to 3-aminophthalate ions with the aid of a catalyst or co-oxidant under alkaline aqueous conditions, producing water, nitrogen gas and light at a wavelength of 425 nm. In this study, cobalt was used for catalysis of luminol-hydrogen peroxide CL.

Marle and Greenway developed a glass microchip for the detection of hydrogen peroxide in rainwater using a luminol-hydrogen peroxide CL system. The sensitive 8 mm-aperture photon detector was aligned to a rectangular channel network. As mentioned earlier, the microchannel did not have a design for improved mixing. In the range from 0.1 to 7.5 μM, the correlation between detected signal and the concentration has a second-order characteristic. The linear range of the characteristics was limited at 0.1 to 1 μM. In the pioneering work by Burdo and Seitz, the maximum CL intensity was between 1 mM and 2 mM, and linearity was observed for lower concentration of H2O2 below 1 mM. This discrepancy can be explained by the lack of a good mixer on the microchip.

In this preliminary study, we investigated the dependency of CL intensity on the flow rate and the length of mixing channel in a passive micromixer based on chaotic advection. Our ultimate objective was to develop a CL LoC system suitable for the determination of analyte at low concentration and with a shorter analytical time. The micromixer design was adopted from Tan et al.’s work, where it was used for mixing of reagents for enzymatic reaction. In the previous work, a low flow rate was used to have a residence time matching the enzymatic reaction time. Since the micromixer is based on chaotic advection, a higher flow rate should result in better mixing. Hence, the potential of fast mixing time of this micromixer was not utilised. In our experiment, a long reaction channel was designed to ensure that the optimum mixing length with a maximum CL intensity could be determined. An optical fiber connected to a photon detector was used to probe the local CL intensity along the channel. The optimum mixing length can then be determined experimentally. Following the optimisation of the flow rate and mixing length, the microchip was used to demonstrate the catalytic interaction between the reactants. The reaction channel has a total length of 202 mm. The estimated total channel volume is about 145 μl. The length of the channel from entrance to the middle point as depicted in Fig. 1 is 111 mm.

Microfabrication

CorelDraw was purchased from Corel Co. (Canada). A commercial CO2 laser system, Universal M-300 Laser Platform, was obtained from Universal Laser Systems Inc. (Arizona, USA). Polymethyl methacrylate (PMMA) sheets of 1 mm thickness were obtained from Ying Kwang Acrylic Trading (Singapore). CorelDraw was used to design the pattern required. The pattern was then transferred to the laser engraving system to make the PMMA parts. The parts were aligned and thermally bonded at a low pressure and a temperature of 170 °C. Tubings were attached by epoxy glue to provide fluidic access to the inlets and outlet to the microchip.

Chemiluminescence reagents

All reagents were analytical grade. The salts were dissolved in ultrapure water (18.2 MΩ cm) and filtered. Luminol was obtained from Fluka (Gillingham, Dorset, UK). Sodium carbonate (NaHCO3) and cobalt(II) nitrate (Co(NO3)2·6H2O) were obtained from Ajax Finechem (Australia). Sodium bicarbonate (Na2CO3) was obtained from GCE Laboratory Chemicals (Germany). 30% (v/v) hydrogen peroxide (H2O2) was obtained from Scharlau (Australia).

Carbonate buffer solution was adjusted to pH 10 by mixing 0.1 M NaHCO3 and 0.2 M Na2CO3 dissolved in deionised water. Luminol was dissolved in this pH 10 buffer solution and filtered with a disposable syringe filter (0.22 μm) to give a concentration of 1 mM. A stock solution of 1 mM cobalt(II) nitrate in deionised water was first made, and then diluted in deionised water accordingly for the actual use. The solution of 30% (v/v)

Materials and methods

Microchip design

As mentioned above, the micromixer design was adopted from Tan et al. The micromixer acts as a reactor, which has a width of 1.8 mm and a depth of 0.4 mm. The staggered herringbone microstructures are designed as pleats with a height of 0.2 mm and width of 0.5 mm. The height of the pleats is exactly half of the channel depth. Unlike the traditional SHM design, this micromixer has two unique features. First, the channel is serpentine reducing the overall foot-print of the microchip. The bending at each corner twists the interface between the two fluids at each junction, and thus further promotes chaotic advection. Second, each period of the channel consists of forward and reversed staggered herringbone grooves. This design reverses the direction of the secondary flow in each period to improve the folding effect on each half of the mixing channel, allowing better catalytic interaction between the reactants. The reaction channel has a total length of 202 mm. The estimated total channel volume is about 145 μl. The length of the channel from entrance to the middle point as depicted in Fig. 1 is 111 mm.

Fig. 1 Schematic layout of the microchip (measurements are in millimetres).
hydrogen peroxide was diluted to 3% (v/v) in deionised water for this experiment and kept in ice before use. All reagents were prepared fresh before the experiments.

Experimental setup

A programmable syringe pump, KD Scientific KDS 250 was purchased from Microdialysis Infusion Pump (Massachusetts, US). A H7467 photon detector with a microcontroller and RS-232C interface was obtained from Hamamatsu (Japan). The data were analysed using Analysis 3.4 was obtained from Vernier Software.

A mixture (1 : 1) of diluted hydrogen peroxide and cobalt(II) solution and luminol in carbonate buffer were delivered from a 5 ml disposable syringe through the syringe pump via Teflon tubing into the microchip. The flow rate in the main channel is twice the flow rate of each inlet. CL light intensity was captured by a 1 mm optical fiber connecting the chip to the adaptor of the photon detector. The experimental setup is shown in Fig. 2.

This photon detector was interfaced directly to a personal computer via a serial connection and data was captured by customized Q-basic programme with an integration time of 30 ms or 50 ms. The data of each reading was obtained over an interval of ten seconds. The data points were subsequently exported to the software Analysis 3.4 for further analysis. Graphs were then plotted using Excel 2007. The counts number was averaged and converted to counts per millisecond. All experiments and readings were conducted in a dark room to eliminate background readings. Each set of experiments were repeated more than 5 times for reliable data statistics. Outliers were removed from the analysis.

Results and discussions

Effect of flow rate on CL intensity

To investigate the effect of the flow rate on the CL intensity emitted from the reactions, an optical fiber was attached at a fixed position of 131 mm from the entrance on the microchannel. A mixture (1 : 1) of 10⁻⁴ M cobalt(II) ions and 3% (v/v) H₂O₂, and luminol in carbonate buffer were introduced steadily via a syringe pump into the microchip, Fig. 2. This system created a continuous flow, producing a constant luminescent light in the microchannel. The flow rates of the main channel were set to increase progressively from 1.67 to 16.7 µl min⁻¹. With a total volume of 145 µl, the corresponding residence time ranges from 1.45 hours to 8.72 minutes. The entrance of the microreactor was taken to be the centre of the T-junction when the fluids first contact each other, Fig. 1. Light emitted from the CL reaction was captured by a flexible, detachable optical fiber and leads to the photon detector. Collected data were analysed in the computer.

Fig. 3 shows the effect of varying flow rate in the channel on the CL intensity emitted from the reactions. A linear correlation was observed between flow rate and CL intensity with \( R^2 = 0.992 \). The general trend is CL intensity emitted from the equivalent concentration of reactants increases with increasing flow rate.

There are several explanations for this phenomenon. A straightforward answer is more reagents are pumped into the microchip at a higher flow rate and therefore a brighter signal is observed. Another explanation is better mixing at high flow rates. The micromixer makes use of chaotic advection. The unique feature about such a micromixer is that a higher flow rate results in the generation of faster vortices, and in turn more vigorous mixing. Therefore, more reactants come to contact and react.

A high flow rate setting is recommended to give a brighter CL signal. A high flow rate also reduces the analytical time required for each sample. However, a high flow rate also leads to the excessive use of reagents. An increase in flow rate also increases the pressure in the microchip leading to leakage. In some cases, an extremely high flow resistance in the channel may cause the syringe pump to stall. Hence, a compromise between high signal magnitude and leakage needs to be reached for an optimal flow rate.

Distribution of CL intensity along the mixing channel

Next, the distribution of CL intensity emitted from the reactions along the mixing channel was investigated for flow rates ranging from 1.67 to 16.7 µl min⁻¹. Using the similar experimental conditions as described previously, the optical fiber was attached at various locations on the chip to measure the light signal. The optical fiber was able to provide local information on the CL reaction along the mixing channel. This information allows monitoring the progress of the reaction along the channel and determining the location of maximum CL intensity.

Fig. 4 shows the CL intensity emitted from the reactions captured along the mixing channel at different flow rates. For

![Fig. 2 Experimental setup.](image)

![Fig. 3 Effect of flow rate on CL intensity at a fixed position (131 mm from the inlet, error bars represent the standard derivations).](image)
each flow rate, the CL intensity from the equivalent concentration of reactants first increases, and then decays along the mixing channel. The maximum CL intensity occurred at around 100 mm from the inlet. A possible explanation for this is that the batch of reagents introduced into the microchip is initially not well mixed. Chemical reactions occurring between the molecules are restricted to the portion of molecules that comes into contact. When the reactants are more homogenized after better mixing downstream, a larger amount of molecules can react leading to a maximum photon emission. Further downstream, the CL intensity drops as the reagents are depleted. As the reaction proceeds over time, these reactants are exhausted leading to a decrease in photon emission.

The trendlines were fitted in the graph of Fig. 4. Using differential calculus, the derivative gradient of the trendline was obtained for each flow rate from 1.67 to 16.7 μl min⁻¹ in the channel. From these derivatives, the position of the location of maximum CL intensity was determined. The flow rate in the channel was converted to velocity by removing the sectional area of the channel. The velocity in the channel was plotted against the position of maximum CL intensity, Fig. 5. A linear trend was observed for the position of maximum CL intensity versus the velocity. The first-order fitting of this relationship is \( L = 27.1v + 99.4 \), where \( L \) is the position of the maximum CL intensity and \( v \) is the average velocity of the fluid flow in the channel, \( R^2 = 0.986 \). The gradient constant of 27.1 represents the reaction time needed for the CL reaction to reach the maximum intensity. This reaction time encompasses both reaction kinetics and the effect of fluid mixing. A minimum length of 99.4 mm is necessary for the reagent to mix, react and emit the most intense CL light.

The details of the effectiveness of this micromixer was investigated in our laboratory but had not been reported. Early studies indicated that the micromixer required a mixing length of 56 mm to achieve mixing efficiency of 90% at 20 μl min⁻¹. This served as a good mixing function in our application. A maximum CL intensity was postulated to have occurred approximately at that position. To our surprise, a further length of 43.4 mm was needed due to the delay in CL emission.

The oxidation of luminol in the CL reaction is a multi-step reaction. Several events may occur in the microreactor progressively. First, the different species of molecules: cobalt(II) ions, hydrogen peroxide and luminol get mixed together. Then, they react by the formation of cobalt-peroxide complex, followed by the oxidation of luminol by one electron to a radical by the cobalt-peroxide complex. And finally, luminol radicals react with hydrogen peroxide to produce the light emission in several reaction steps.

Therefore, the length of micromixer can be shortened to 60 mm to provide the need for mixing. And the reaction channel can be lengthened by 40 mm to cater for the relay in CL emission. The detector needs to be placed away from the entrance at 100 mm. This is important because the fabrication of intricate microstructures in the microchannel can be time-consuming. These adjustments in the design will aid in the fabrication of future microchip, because it will reduce redundant features.

Using the relationship obtained earlier, the location of the maximum CL intensity along the mixing channel can be adjusted. For instance, to align the maximum CL intensity to the centre of the mixing channel at 110 mm, the velocity in the channel is estimated to be 0.39 mm s⁻¹, corresponding to a flow rate of 16.8 μl min⁻¹. Since the optical fiber can only collect the local intensity of the light signal, it is important to know the precise location of the maximum CL intensity.

**Effect of concentrations of cobalt(II) ions on CL intensity**

Next, the effects of the concentrations of cobalt(II) ions on CL intensity emitted from the reactions was investigated. The concentration of cobalt(II) ions ranges from \(10^{-10}\) to \(10^{-3}\) M. A mixture (1 : 1) of cobalt(II) ions and 3% (v/v) H₂O₂, and 1 mM luminol in carbonate buffer were introduced into the microchip. The flow rate was set to 16.8 μl min⁻¹. The minimum analytical time for each sample was 8.65 minutes. The optical fiber was placed at 110 mm from the entrance at 60 mm to provide the need for mixing. And the reaction channel length of 99.4 mm is necessary for the reagent to mix, react and emit the most intense CL light.

The calibration graph for cobalt(II) ions against CL intensity was plotted in Fig. 6. The CL intensity increased with increasing concentration of cobalt(II) ions. The linear range was observed.
from $10^{-10}$ to $10^{-3}$ M. The fitting function is $y = 1680x + 155$, $R^2 = 0.998$, where $y$ and $x$ represent the CL intensity in counts and the concentration of cobalt(II) ions in mM. Deionised water was used in place of cobalt(II) ions, which gave a background (blank) of 0.24 ± 0.28.

The limit-of-detection (LOD) was calculated from the y-intercept plus three times the standard deviation of the regression (SD x/y). The calculated LOD was 1.06 nM. The differences in the number of counts in CL intensity between $10^{-10}$ and $10^{-3}$ M were insignificant to produce appreciable differences for the measurement of cobalt(II) ion concentration. Hence, the useful linear range of the microchip would be $10^{-3}$ to $10^{-7}$ M.

Luminol CL reaction can be catalysed by various metal ions. Cobalt(II) ion is known to be a more effective catalyst for this reaction than other ions such as copper(II), nickel(II), iron(III), iron(II) and manganese(II). The applications of the microchip would also be useful for the measurement of water quality for the presence of heavy metals such as cadmium(II) and other ions. Assessing the level of heavy metals contamination is important to monitor pollution from industrial wastewater, safety of food, including agricultural and fishery products in marine coastal water.

**Effect of concentrations of hydrogen peroxide on CL intensity**

The detection and determination of hydrogen peroxide is another important application of a luminol CL system. The CL intensity given off by different concentrations of hydrogen peroxide was studied. The concentration of cobalt(II) ions was fixed at $10^{-5}$ M.

The effect of the concentration of hydrogen peroxide from $1.47 \times 10^{-7}$ to $1.47 \times 10^{-3}$ M on CL intensity was explored. From $1.47 \times 10^{-6}$ to $2.9 \times 10^{-4}$ M of hydrogen peroxide, CL intensity of the reaction increased linearly with increasing concentration of hydrogen peroxide, Fig. 7. The observed linear response was similar to the pioneering work of Burdo and Seitz. The linear fitting function was $y = 29600x + 8.28$, where $y$ is the CL intensity in counts, and $x$ is the concentration of hydrogen peroxide in mM, $R^2 = 0.999$. The background (blank) for this experiment was determined as $8.28 \pm 0.72$ using deionised water in place of $\text{H}_2\text{O}_2$.

The limit-of-detection (LOD) was calculated from the concentration of the analyte required to give a signal equal to the background (blank) plus three times the standard deviation of the blank. The calculated LOD was 73.1 nM. At $1.47 \times 10^{-7}$ M of hydrogen peroxide, the CL signal was nearly indistinguishable from the background. Hence, the lowest reliable limit for determining hydrogen peroxide was $1.47 \mu\text{M}$ for this system.

The linear correlation implies that this microchip can be used for the determination of the concentration of hydrogen peroxide. Hydrogen peroxide is produced in oxidative stress in cells. Hydrogen peroxide is a product from the oxidation of glucose, cholesterol and uric acid by oxidase enzyme. Hence, the detection of hydrogen peroxide is useful for medical diagnostics. Hydrogen peroxide can also be used to monitor rainwater, which is an efficient oxidiser of sulfur dioxide to produce sulfuric acid, an important compound in acid rain formation.

**Further optimization of CL intensity in the microchip**

The local measurement using the optical fiber is only able to sample a small amount of reactant volume in the microchannel. Therefore, the CL intensity captured from the reaction was weak. Higher flow rate set will increase the CL intensity. Given a flow rate of 100 $\mu\text{L}$ min$^{-1}$, the CL intensity at the position of 131 mm from the inlet is about 1670 counts for the concentration of $10^{-3}$ M cobalt(II) ions. The analytical time for each sample was about 1.5 min, which is a relatively short time. However, the high flow rate setting is limited by the amount of analytical sample, bonding strength of the microchip, and fitting of the tubing.

The CL intensity emitted from the reaction can be increased by increasing the reactant volume for a fixed concentration. To put on trial, the optical fiber is attached to the plug outlet on the microchip for the measurement of CL intensity emitted. The plug outlet holds an approximate volume of 0.5 mm$^3$. The flow rate was 163 $\mu\text{L}$ min$^{-1}$ in the main channel. The analytical time for each sample was further reduced to about 1 min. This flow rate would push the maximum CL intensity to the end of the reaction channel. A linear range of $10^{-9}$ to $10^{-5}$ M was obtained between the concentration of cobalt(II) ions and CL intensity, following
the fitting line of \( y = 19450x + 89.1 \), where \( y \) and \( x \) represent the CL intensity in counts and the concentration of cobalt(II) ions in mM, \( R^2 = 0.995 \). Based on the line gradients, this method can be 11 times more sensitive than attaching the optical fiber at the location of 110 mm. Hence, future work should aim to enlarge this reactant volume to expose more CL light to the photon detector. The detection limit and sensitivity may be lowered further by increasing the sampling reactant volume. Such microchip design may include a shorter micromixer and a spiral channel detection system.\(^{11}\)

**Conclusion**

A microchip, containing a passive micromixer as the reactor was characterized for luminol-peroxide chemiluminescence (CL) detection. A high flow rate was found to lead to a brighter CL signal intensity and shorter analytical time. A second-order distribution of CL intensity was found along the mixing channel. The maximum CL intensity of this intensity profile shifted with increasing flow rate. The concentration of cobalt ions or hydrogen peroxide correlated with CL intensity emitted from the reactions. Larger reactant volume was important for a more intense CL signal to increase its sensitivity. This approach seems to be suitable for designing a LoC for CL applications.

**References**

18. X. Liu, A. Li, B. Zhou and C. Q. H. Ren, *Chem. Cent. J.*, 2009, **3**.