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# Chemiluminescence microfluidic chip fabricated in PMMA for determination of benzoyl peroxide in flour

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**Abstract**

A rapid and sensitive chemiluminescence microfluidic chip fabricated in polymethyl methacrylate for determination of benzoyl peroxide in flour is described. The width of the microchannel was 200 μm and the depth was 100 μm in the chip (50 × 40 × 5 mm). The total length of the microchannel was 67 mm with the detection area of 5 × 5 mm. Benzoyl peroxide can directly oxidize luminol to produce chemiluminescence. Two injectors of double-tee injector and microvalve injector were used. The sampling volume was 0.16μl for the double-tee injector and 0.25μl for the microvalve injector. The linear range of the benzoyl peroxide concentration was $8 \times 10^{-7}$–$1 \times 10^{-4}$ g/ml. The detection limit was $4 \times 10^{-7}$ g/ml and the R.S.D for eight times is 3.06% for the double-tee injector and 2.41% for the microvalve injector. The proposed method has been successfully applied to the determination of benzoyl peroxide in flour.


*Keywords:* Microfluidic chip; Benzoyl peroxide; Chemiluminescence

## 1. Introduction

Benzoyl peroxide (BP) is a common additive in flour because of its bleaching and sanitizing properties. In the bleaching process, flour is exposed to benzoyl peroxide to whiten and brighten flour colour (Fennema, 1985). Benzoyl peroxide could affect baking quality and excessive benzoyl peroxide in flour could induce allergic reactions, weak cancer causing, vitamin E and nutrients destruction. In US and UK, the maximum concentration regulated at BP is 0.05 g/kg. In our country, the maximum concentration regulated at BP is 0.06 g/kg. Rapid, sensitive and selective methods for the determination of benzoyl peroxide are required in the food and clinical fields.

Recently, many analysis protocols have been utilized, including HPLC (Akasaka, Ohrui, & Meguro, 1993; Onishi, Yomota, Sugimoto, Kubota, & Tanamoto, 2004; Saiz, Manrique, & Fritz, 2001; Wada et al., 2003), spectrophotometry (Mori, Tominaga, Fujita, & Matsuo, 1997), ion chromatography (Chen, Mou, Hou, & Ni, 1998). However, HPLC with the UV detector (Saiz et al., 2001; Wada et al., 2003) is less sensitive. The HPLC with the fluorescence detection has a tedious process for the sample. Chemiluminescence has been used for the determination of lipid hydroperoxide (Miyazawa, Fujimoto, & Kaneda, 1987; Wada et al., 2003). In the latter paper, Miyazawa reported that chemiluminescence can be generated during the oxidation of luminol by the reaction with hydroperoxide and cytochrome *C* under mild conditions including benzoyl peroxide. The sensitivity of benzoyl peroxide is low. Wada et al. (2003) reported a HPLC method with peroxyoxalate chemiluminescence detection for determining

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BP in flour. Recently, our group Yang, Zhang, and Hun (2004) reported a high sensitivity microliter size chemiluminescence system for determination of BP in flour. In that system, the chemiluminescence reagent must be changed after 30–50 runs and the instrument must be turned off.

After the concept of micro total analysis system (μTAS) was introduced by Manz, Graber, and Widmer (1990), the area of μTAS has been growing rapidly in the recent years. Because miniaturization is currently generally recognized as one of the most important trends in the development of analytical instrumentation, the ultimate purpose of μTAS is the integration of the entire analytical process on a micro scale device (Becker & Locascio, 2002; Chabinye et al., 2001; Fu, Yang, Lee, & Liu, 2002; Liu, Ozaki, Utsumi, Hattori, & Terabe, 2003). Chemiluminescence (CL) is a promising method of detection for micro-total analytical system (Greenway, Nelstrop, & Port, 2000; Greenwood, Merrin, McCreedy, & Greenway, 2002; Lv, Zhang, & Chen, 2003; Xu, Bessoth, Eijkel, & Manz, 2000) since no light source is required. Luminol and $H_2O_2$ are the most commonly used CL reagents, however $H_2O_2$ can easily produce gas bubbles which will affect the analysis. In this work, benzoyl peroxide can directly oxidize luminol to produce chemiluminescence resulting the high sensitivity. As no $H_2O_2$ is used, no gaseous products develop and this contributes to the reproducibility. Using the easy, fast and sensitive chemiluminescence system on the microfluidic chip for determination of benzoyl peroxide is feasible. The microfluidic chip was fabricated in polymethyl methacrylate (PMMA) and the microchannel was obtained by $CO_2$ laser ablation. A double-tee injector structure and a microvalve injector structure were used. The sampling volume was 0.16 μl for the double-tee injector structure and 0.25 μl for the microvalve injector. The linear range of the benzoyl peroxide concentration was $8 \times 10^{-7}$–$1 \times 10^{-4}$ g/ml. The detection limit was $4 \times 10^{-7}$ g/ml and the R.S.D for eight times is 3.06% for the double-tee injector and 2.41% for the microvalve injector. The proposed method has been successfully applied to the determination of benzoyl peroxide in flour.

## 2. Materials and methods

### 2.1. Chemicals

All chemicals were of analytical reagent grade; doubly distilled water was used for the preparation of solutions. A 0.01 M luminol solution was purchased from Kangpei Technology Co. (Xi'an, China, 98%), which was prepared by dissolving 0.1772 g of luminol in 100 ml of 0.01 M NaOH solution and was used after storing at room temperature for a week. A $10^{-3}$ g/ml stock solution of benzoyl peroxide was prepared by dissolving 0.05 g of benzoyl peroxide in 50 ml ethanol, which was purchased from Shanghai Caoxing Zhongli Chemical Regent Co. (Shanghai, China). The stock solution can be used for 2 days at room temperature and working solutions were prepared freshly from the stock solution by dilution with water. Ethanol was purchased from Xi'an Chemical Reagent Co. (Xi'an, China)

### 2.2. Instrumentation

A schematic diagram of the CL microfluidic system is shown in Fig. 1. A homemade CL microfluidic chip fabricated in PMMA (Shandong Zhangqiu organic glass Co., China) was coupled with the injection pumps (Ruimai Electronics Co., China) by the PTFE tubing (0.2 mm i.d, 0.5 o.d). The holes (Fig. 1.) on the top plate were connected with PTFE tubing with epoxy. The photomultiplier tube (PMT) (Hamamatsu, Japan) was placed under the chip. The signal produced by the CL microfluidic chip was detected and recorded by a computerized luminescence detector (Ruimai Electronics Co., China). Data acquisition and treatment were performed with luminescence detector software running under WINDOWS 98. The clamps used here were purchased from the supermarket which can also be used to clamp the books. Absorbance monitoring was done using a spectrophotometer Model-721(Shanghai Third Analytical Instrument Co., China).

### 2.3. Fabrication of the microfluidic chips

The chip with double-tee injectors was fabricated from two transparent PMMA plates ($50 \times 40 \times 5$ mm).

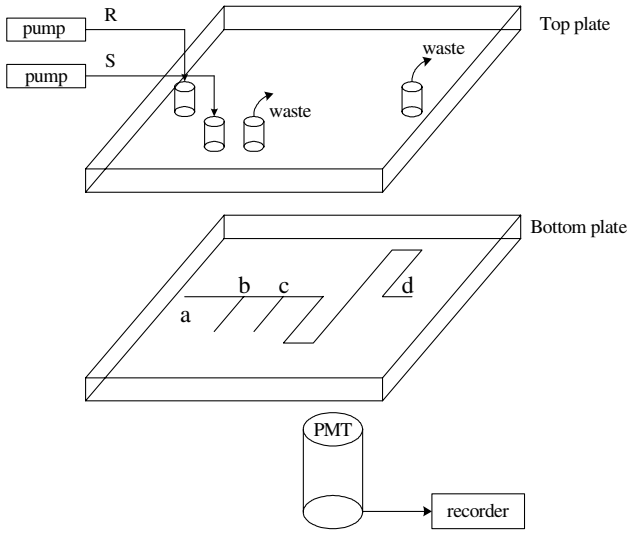


Fig. 1. Schematic diagram of CL microfluidic chip with the double-tee injectors. The width of the microchannel: 200 μm; the depth: 100 μm. *ab* = 9 mm, *bc* = 8 mm, *cd* = 22 mm. R, CL reagent; S, sample.

The top plate had four drilled holes in it with 0.5 mm i.d. In Fig. 1, two holes on the top plate were linked with the injection pumps. Two holes were for the waste. Microchannels were fabricated by laser ablation on the surface of the bottom plate. On the bottom plate, the width of the microchannels was 200 μm and the depth 100 μm. The total length of the microchannels was 67 mm of which the length of the separation was 50 mm. Finally, the bottom plate was bonded to the top plate by heating at 108 °C for 10 min with 1.5 MPa. The detection area was $5 \times 5$ mm on the microchannel and the distance to *d* was 3 mm. After bonding the two plates, the holes linked the microchannels on the bottom plate with the injection pumps by the PTFE tubes.The black dope shaded its bottom plate except for the detection area.

The fabrication of the microvalve can also be seen in Fig. 4. The material of the microvalve was polytetrafluoroethylene (PTFE) and it was made by our group. It was fixed on the chip by the screws and used electromotion switch for the sampling. The chip only had the microchannels of *cd* in Fig. 1. The PTFE tubes were used to link the pumps, the microvalve and the microchannels together.

### 2.4. Sample preparation

A $10^{-3}$ g/ml stock solution of benzoyl peroxide for calibration solutions was prepared by dissolving 0.05 g of benzoyl peroxide in 50 ml ethanol. Calibration solutions were prepared from the stock solution by appropriate dilution with water.

We used wheat milled to flour without pollution for the blank sample. A 25-ml volume of ethanol was added to the flour (3.0000 g) (Cai & Zhang, 2002). The suspension was sonicated for 10 min and centrifuged for 30 min. The supernatant was divided into two parts. One part of 50 μl was used for the microfluidic chip with CL determination. The other part was used for the spectrophotometry determination. Benzoyl peroxide can oxidize $Fe^{2+}$ to $Fe^{3+}$. We used the discoloring reaction between phenanthroline and $Fe^{3+}$ to determination the benzoyl peroxide.

## 3. A sample injection

The sample injection schematic is shown in Fig. 2. In the first step the injection pump introduced sample from channel C to B and channel A was closed using a clamp. We used the clamp on the PTFE tube which connected the pump and the chip. The microchannel behind D has four corners which provide sufficient resistance. Without clamp so that the solution will be introduced from C to B and not be introduced to D. Thus a well-defined sample plug was formed shown in the photo of Fig. 3. In the second step, channels B and C were closed by a clamp and luminol was pumped through channel A moving the sample plug to the detection area.

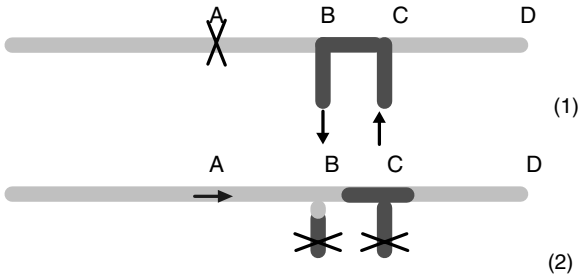


Fig. 2. Sample injection schematic. In the first step, A is closed and sample is introduced between B and C. In the second step B and C are closed and solvent is pushed through A moving the sample between B and C into the detection area.

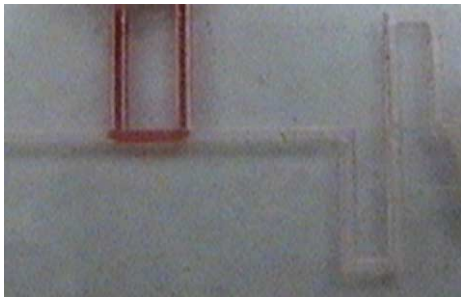

Fig. 3. Photo of red ink as sample plug introduced with double T structure.

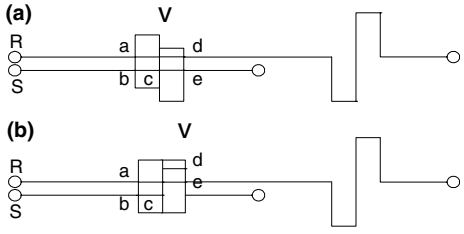


Fig. 4. Schematic diagram of CL microfluidic chip with microvalve injectors. R, reagent; S, sample; (A) sample loading; (B) sample injection.

From the photo a well-defined sample plug without diffusion can be seen. The length of the sample plug was 8 mm and the sampling volume 0.16μl every time. The clamps used here were very tight and this contributed to the reproducibility.

After the samples were injected into the microchannel, the injection pump connecting with luminol started. Sample mixed with luminol in *cd* and a CL signal was produced. The concentrations of benzoyl peroxide were quantified by using the CL intensity.

For the microvalve sampling, the microvalve was made by ourselves. It was fixed on the microfluidic chip by two small screws and connected the microchannels.

When the microvalve is in the "loading" position (A) in Fig. 4., the injection pump injected the sample from the microchannel to the microvalve through a PTFE tube. The sample volume filled in *ce* was 0.25 μl (length of *ce* 5 mm, $d = 0.25$ mm). The excess sample was discarded through the waste. When the microvalve was switched to the "injection" position (B), *e* was connected to *a* and the sample plug in *ce* was injected into the reagent solution of luminol. Sample diffused into the solution of luminol in the microchannel behind and produced chemiluminescence. The detection area was the same as with the double-tee injectors.

## 4. Results and discussion

### 4.1. Comparison of the two injectors

For the double-tee injection, we first opened the pump connected with the sample microchannel. When the sampling microchannel was filled using the clamp, we opened the other pump connecting the luminol solution. Sample merged with the luminol to produce CL. For the microvalve injection, sample first filled in it. After the microvalve was switched to connect with the luminol solution, we could detect CL later.

Eight consecutive injections are shown with the two tested injectors in Fig. 5. The relative standard deviation for $6 \times 10^{-6}$ g/ml BPO of the double-tee injectors was 3.06% ($n = 8$) and of the microvalve injectors 2.41% ($n = 8$).

### 4.2. Influence of luminol concentration

The luminol concentration was varied from $1 \times 10^{-4}$ to $1.5 \times 10^{-3}$ mol/l as shown in Fig. 6. On increasing the luminol concentration from $1 \times 10^{-4}$ to $1.5 \times 10^{-3}$ mol/l, the CL intensity was found to increase with the luminol concentration until a maximum was reached at $5 \times 10^{-4}$ mol/l luminol. When the luminol concentration increased further, the CL intensity decreased and reached a plateau. Consequently, concentration of $5 \times 10^{-4}$ mol/l luminol was selected for subsequent analysis.

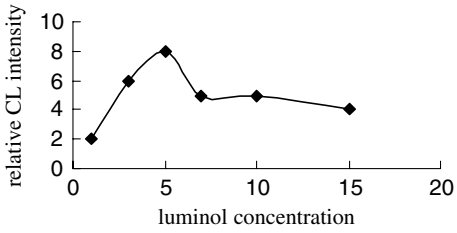


Fig. 6. Effect of luminol concentration ($\times 10^{-4}$ mol/l) on chemiluminescence intensity in microchip.

### 4.3. Influence of the flow rate

Due to the small solvent volume in the microfluidic chip, the mixing and reaction time were fast. Selection of a proper flow rate for sufficient mixing of the solvents to achieve optimal CL intensity. With the double-tee sampling structure (Fig. 1.), the flow rate of the injection pump on the CL response was investigated from 0.01 to 0.1 ml/min which was shown in Fig. 7(a). From the CL intensity, we selected 0.05 ml/min with the highest CL intensity. If the flow rate was faster, the solvents could not mix sufficiently. If the flow rate was slower, injector diffusion lead to a wide peak.

With the microvalve injector, 0.06 ml/min was found to provide optimal CL intensity which was shown in Fig. 7(b).

### 4.4. Influence of buffers and pH

Three different buffers were studied. They were $Na_2CO_3$–$NaHCO_3$ (pH: 9.2–11.0), $NH_3$–$NH_4Cl$ (pH: 9.0–10.0)and $Na_2HPO_4$–NaOH (pH:11.0–12.0). It was found that in the pH range of 11.0–12.0 the CL intensity was of the highest and reproducibility was optimal. The influence pH on the CL intensity was investigated in the range of 11–12 as shown in Fig. 8. At pH 11.5, the CL intensity reached a maximum. This pH was selected for further experiments.

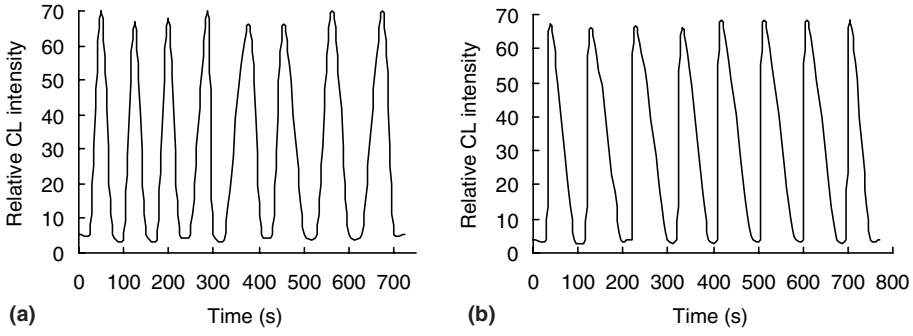


Fig. 5. Precision of the two sampling structures: (a) the double-tee injector structure ($n = 8$); (b) the microvalve injector structure ($n = 8$).

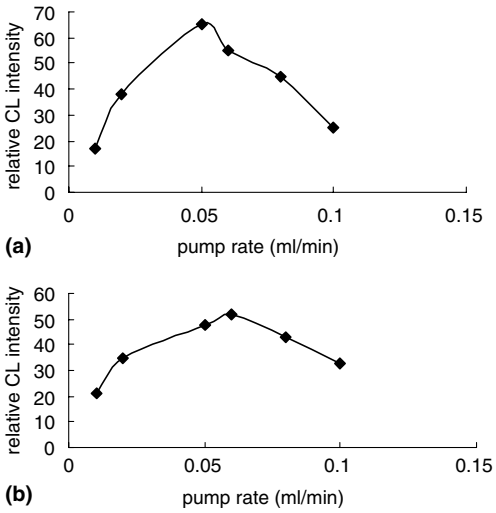


Fig. 7. (a) Effect of pump rate on chemiluminescence intensity of the double-tee injector on the microfluidic chip. (b) Effect of pump rate on chemiluminescence intensity of the microvalve injector on the microfluidic chip.

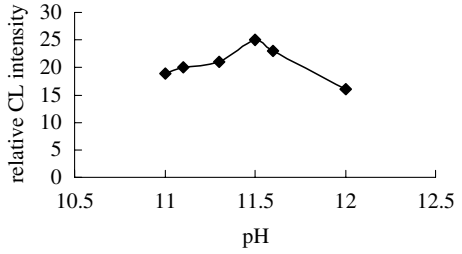


Fig. 8. Effect of pH on chemiluminescence intensity on the microfluidic chip.

### 4.5. Benzoyl peroxide measurements

Under the selected conditions, the response to the benzoyl peroxide concentration was linear over a range of $8\times10^{-7}$–$1\times10^{-4}$ g/ml with a regression equation of $I = 2.5\times10^{5}$ C(g/ml) + 9.5 ($r^2 = 0.9966$, $n = 7$) and the detection limit was $4\times10^{-7}$ g/ml ($3\sigma$).

The influence of interferences were investigated by analyzing a standard solution of $4\times10^{-6}$ g/ml benzoyl peroxide to which increasing amounts of interfering compounds were added. The tolerable limit of a foreign species was taken as a relative error not greater than 5%. The tolerated ratio of foreign substances to $4\times10^{-6}$ g/ml was 1000 for ethanol, faecula, $Na^+$, $K^+$, $CO_3^{2-}$, $PO_4^{3-}$, $VB_1$, maltose, glycine, arginine, serine, theonine, fructose, glucose; 100 for $Pb^{2+}$, $Cr^{3+}$, $Fe^{3+}$, $S^{2-}$, $VB_2$, praline; 20 for $Co^{2+}$, $Ni^{2+}$, $Cd^{2+}$; 5 for $Cu^{2+}$, 1 for $Fe^{2+}$. $Fe^{2+}$ can catalyze CL reaction of luminol with dissolved oxygen. And the sample was dissolved in the ethanol and the metal ions cannot be dissolved in the ethanol. So $Fe^{2+}$ can not interfere with the determination of benzoyl peroxide in flour.

Table 1
Results of analysis of benzoyl peroxide in flour

| Sample | Proposed method (g/ml) | Spectrophotometry (g/ml) |
|---|---|---|
| 1 | $3.0\times10^{-6}$(±3.0%) | $3.2\times10^{-6}$(±3.5%) |
| 2 | $3.7\times10^{-6}$(±2.9%) | $3.6\times10^{-6}$(±3.2%) |
| 3 | $5.7\times10^{-6}$(±2.8%) | $5.5\times10^{-6}$(±2.8%) |
| 4 | $1.6\times10^{-6}$(±3.1%) | $1.9\times10^{-6}$(±3.8%) |
| 5 | $1.0\times10^{-6}$(±3.2%) | $1.2\times10^{-6}$(±3.7%) |
| 6 | $8.7\times10^{-6}$(±3.7%) | $9.0\times10^{-6}$(±4.0%) |
| 7 | $1.5\times10^{-5}$(±3.7%) | $1.4\times10^{-5}$(±3.1%) |
| 8 | $2.6\times10^{-5}$(±3.8%) | $2.6\times10^{-5}$(±4.7%) |
| 9 | $6.0\times10^{-5}$(±3.6%) | $5.8\times10^{-5}$(±3.7%) |

Average of three replicates (±R.S.D).

The microfluidic chip was applied to the determination of benzoyl peroxide in flour. The sample preparation can be seen in 2.4. The results were given in Table 1. As can be seen, the results with the present method agreed with those obtained by spectrophotometry.

## 5. Conclusion

We have described the successful use of a CL microchip fabricated in PMMA. The technology makes use of both the high selectivity and sensitivity of CL detection. In this method no separation or preconcentration steps are needed. The linear range for benzoyl peroxide determination is $8\times10^{-7}$–$1\times10^{-4}$ g/ml and the detection limit is $4\times10^{-7}$ g/ml. The reagent consumption is low. Consequently, the microchip analysis system has great potential for application in food.

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