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# Microanalysis System Based on Electrochemiluminescence Detection

Hiroki Hosono, Wataru Satoh, Junji Fukuda and Hiroaki Suzuki\*

Graduate School of Pure and Applied Sciences, University of Tsukuba,  
1-1-1 Tennodai, Tsukuba, Ibaraki 305-8573, Japan

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An electrochemical analysis system with microfluidic and sensing functions was developed. The microfluidic components consisted of a reaction chamber and hydrophilic flow channels with valves. The valves were formed as gold electrodes and were operated on the basis of direct electrowetting. A working electrode used to generate electrochemiluminescence (ECL) was formed in the reaction chamber. A solution that filled two injection ports could be injected into the reaction chamber simultaneously by opening the valves. When a solution containing an amino acid and a reagent solution containing  $\text{Ru}(\text{bpy})_3^{2+}$  were mixed and a positive potential was applied to the electrode to generate ECL, red luminescence was observed. The luminescence intensity, measured using a photodiode, increased with increasing solution pH. The luminescence intensity also increased with increasing concentration of the amino acid. The lower detection limits were on the order of lower pM levels, and the scattering of the detected values of concentration increased as the concentration approached the detection limits.

## 1. Introduction

With the progress of the micro-total-analysis system ( $\mu\text{TAS}$ ) technology, the number of promising applicable fields is steadily increasing, and highly sophisticated devices have been proposed.<sup>(1–4)</sup> For the detection of analytes, representative methods used for the microsystems have often been the detection of absorption and fluorescence. However, a problem often reported with the former has been the optical path length, which is very short in microflow channels. Compared with the detection of absorption, fluorescence detection is more advantageous in collecting light emitted from a very small space. However, a problem is the necessity of a light source, which is usually bulky,

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\*Corresponding author: e-mail: hsuzuki@ims.tsukuba.ac.jp

and the difficulty in achieving a very low detection limit due to the background light. Compared with these methods, a promising alternative is electrochemical luminescence (ECL).<sup>(5-7)</sup> An advantage compared with absorption or fluorescence detection is that no light source is required; only a working electrode is necessary to generate ECL. This is very attractive for promoting the integration of components, because the electrode can be formed as a thin- or thick-film pattern using microfabrication techniques, and a detector can additionally be formed by conventional semiconductor technology. Furthermore, detection without a light source has a significant effect on reducing the background signal and lowering the detection limit.

When conducting ECL detection, a necessary procedure is the mixing of a sample solution with a solution containing a luminophore. To this end, we used a microfluidic transport system based on capillary action and controlled by electrowetting. Both the capillary action and electrowetting are phenomena observed as an effect of the interfacial tension between the solid / liquid interface. This is very promising for achieving highly efficient transport of solutions with minimum energy consumption. In the electrowetting, the wettability of a metal surface is controlled by changing the electrode potential.<sup>(8)</sup> Therefore, electrowetting is particularly attractive for active microfluidic transport.<sup>(9-11)</sup> In this study, the function of our device was checked by determining the concentration of L-amino acids. A solution containing the amino acid and a luminophore could be transported and mixed in a reaction chamber by opening valves in the flow channels. Using a working electrode for the ECL, red luminescence was observed and the concentration of amino acids could be determined. The fabrication and characterization of the device are presented in this paper.

## 2. Experimental

### 2.1 Reagents and materials

Reagents and materials used for the fabrication and the characterization of the device were obtained from the following commercial sources: thick-film photoresist, SU-8 2100, from Micro Chem (Newton, MA, U.S.A.); negative photoresist, OMR-83, from Tokyo Ohka Kogyo (Kawasaki, Japan); precursor solution of polydimethylsiloxane (PDMS), KE-1300T, from Shin-Etsu Chemical (Tokyo, Japan); tris(2,2'-bipyridyl)dichlororuthenium(II) hexahydrate, from Sigma-Aldrich Japan (Tokyo, Japan). The other reagents were of analytical-reagent grade and obtained from Wako Pure Chemical Industries (Osaka, Japan). All solutions were prepared using distilled deionized water. Standard solutions and reagent solutions were prepared using the following buffer solutions containing 10 mM KCl: 0.1 M  $\text{KH}_2\text{PO}_4\text{-NaOH}$  (pH 7.0 and 8.0), 0.1 M  $\text{H}_3\text{BO}_3\text{-NaOH}$  (pH 9.0 and 10.0), and 0.1 M  $\text{K}_2\text{HPO}_4\text{-NaOH}$  (pH 11.0 and 12.0).

### 2.2 Construction of the microsystem

Figure 1 shows the fabricated device. Three-electrode systems for the actuation of the valves and the generation of ECL were formed in the reaction chamber. The electrodes were formed by sputtering the necessary metals and patterning them by chemical etching or lift-off. The active areas of the electrodes and the flow channel areas were delineated

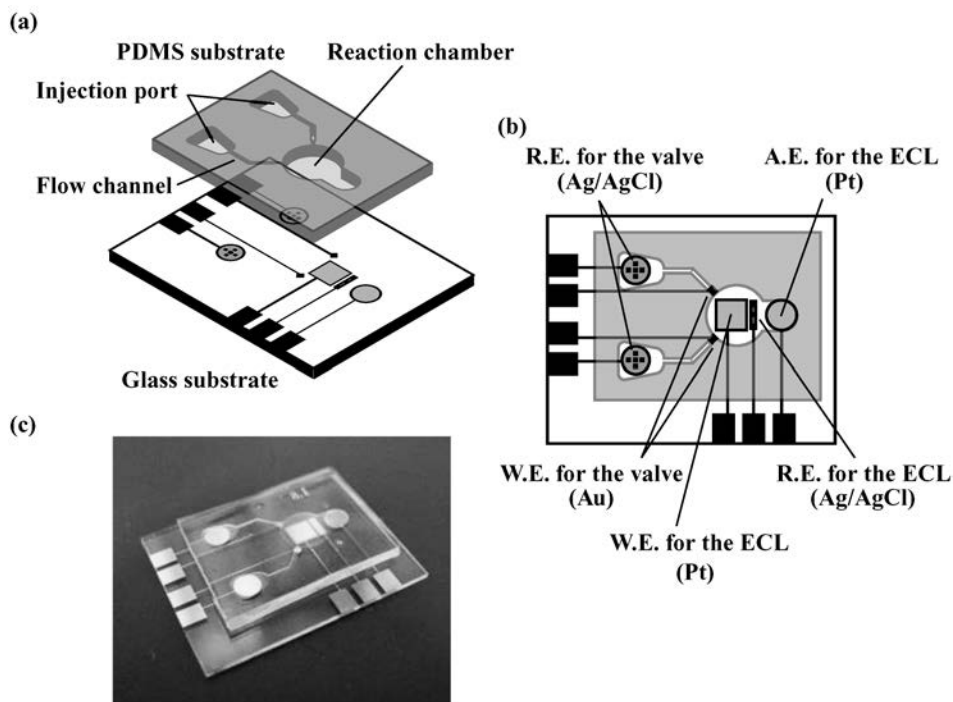


Fig. 1. Microsystem with microfluidic and sensing functions. (a) Decomposed structure. (b) Top view showing the relation between the electrodes and the flow channels. (c) Completed device. W. E., working electrode; R. E., reference electrode; A. E., auxiliary electrode.

with the negative photoresist. For the generation of the ECL, Pt was used for the working and auxiliary electrodes and Ag/AgCl was used for the reference electrode. The active area of the working electrode was  $1.5 \text{ mm} \times 1.5 \text{ mm}$ . In the two flow channels, a gold working electrode was formed as the valve. In forming the Ag/AgCl electrodes, a constant current ( $20 \mu\text{A}$ ) was applied for 1 min in a 0.1 M KCl solution ( $25^\circ\text{C}$ ).

Flow channels and a reaction chamber were formed with PDMS. In forming the structure, a template was first formed using the thick-film photoresist. A precursor solution of PDMS was then poured onto the template. After PDMS was cured, it was peeled off from the template. As a result, an accurate replica of the template was obtained. The reaction chamber was circular with a diameter of 4 mm and a depth of 80  $\mu\text{m}$ . The width and depth of the flow channels were 300  $\mu\text{m}$  and 80  $\mu\text{m}$ , respectively. The transport efficiency in the flow channels may be improved by oxidizing the surface of the PDMS structures by a method such as oxygen- or air-plasma treatment. However, this was not carried out in this experiment, because the treatment often makes normal function of the valves difficult. On the other hand, the glass surface exposed in the

flow channel was made sufficiently hydrophilic. During fabrication, the glass area seemed to be contaminated by organic residues and was not sufficiently hydrophilic after the formation of the electrode structure. As a result, solutions that filled the injection ports did not proceed at all. To solve the problem, the glass substrate with the electrode patterns was immersed in a solution containing 13 wt.% sulfuric acid and 54 wt.% hydrogen peroxide at room temperature. The process recovered the original hydrophilicity of the glass, and the solutions then permeated into the flow channel spontaneously. The patterns of the electrodes, flow channels, and the reaction chamber were aligned and the two substrates were fixed. A photodiode (S1226-18QB, Hamamatsu Photonics, Shizuoka, Japan) was fixed in the PDMS substrate over the working electrode for the generation of the ECL.

### 2.3 Structure and operation of the valves

Figure 2 shows the structure of a valve and how it works in the flow channel. As mentioned earlier, a gold working electrode is an essential component of the valve. In this area, the three walls formed from PDMS are hydrophobic, whereas the glass area at the bottom of the flow channel is hydrophilic. Therefore, a motive force is generated only on the hydrophilic glass area. However, as the width of the flow channel increased, the motive force on the gold electrode increased, even when the electrode was switched off and the surface was not sufficiently hydrophilic. As a result, the valve could not stop the solution column completely. Therefore, in the presented microsystem, the valve area was narrowed ( $150\ \mu\text{m}$ ) to increase the resistive force on the PDMS walls. This effectively prevented the solution from moving spontaneously when the valve was switched off.

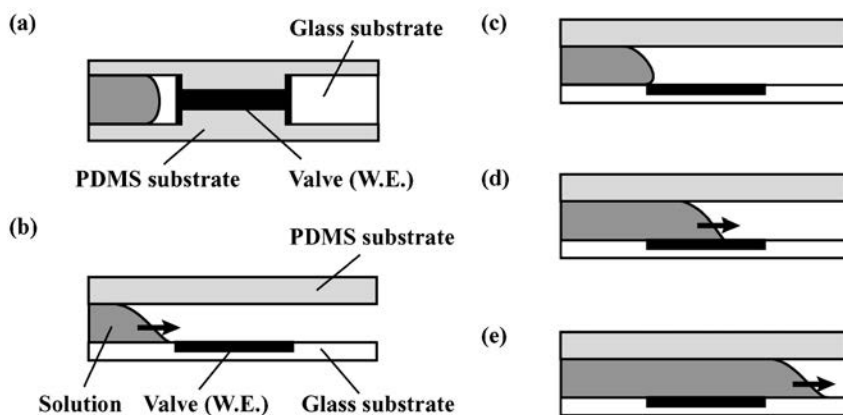


Fig. 2. Details of the structure of the valve area and operation of the valve. (a) Top view of the valve area. (b) Cross section of the same area along the flow channel showing the solution passing along the hydrophilic glass area. (c) Solution stopped at the valve. (d) Solution crossing the valve area. (e) Solution passing along the hydrophilic glass area.

Figures 2(b)–2(e) also show the function of the valve. Since the flow channels on both sides of the valve are hydrophilic as a whole, a solution in the injection ports is transported on the glass area of the flow channel by capillary action (Fig. 2(b)) and stops at the edge of the gold electrode (Fig. 2(c)). Once the valve is switched on, the solution column passes the valve area (Fig. 2(d)) and is again transported on the glass area of the flow channels (Fig. 2(e)). In operating the valve, it is sufficient to apply the potential only during the passage of the solution through the valve area. Therefore, power consumption can be minimized.

There has been a controversy in the formulation of theories on the dynamic mechanism of electrowetting.<sup>(12–14)</sup> However, a common point is that the accumulation of charges occurs in the vicinity of the contact line resulting in the generation of an electrostatic force which pulls the contact line outward. It is apparently observed as a change in the interfacial tension between the electrode and the solution.

#### 2.4 Principle of detection

The ECL is emitted as a result of a series of reactions involving  $\text{Ru}(\text{bpy})_3^{2+}$  as shown in Fig. 3.<sup>(15–17)</sup> First,  $\text{Ru}(\text{bpy})_3^{3+}$  is produced by oxidizing  $\text{Ru}(\text{bpy})_3^{2+}$  on the platinum working electrode.

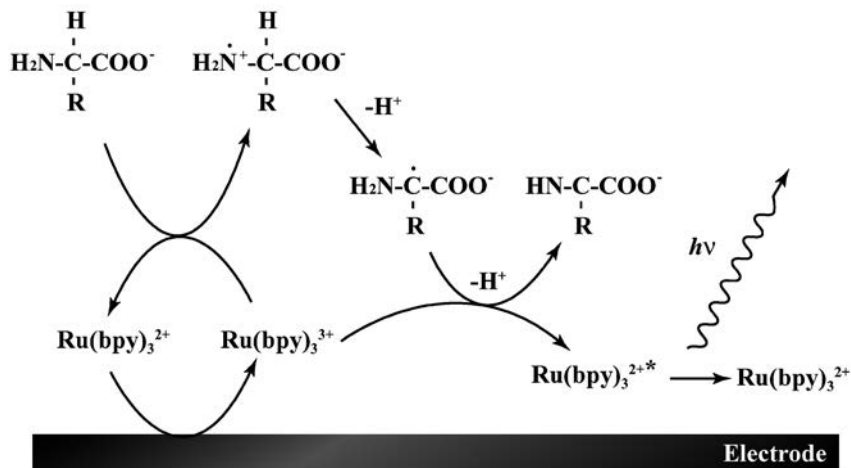
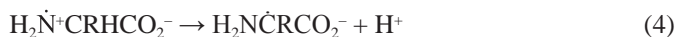
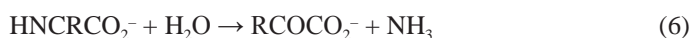


Fig. 3. Reaction scheme of the ECL.

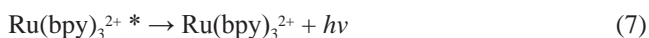
Radical anions are formed in the reaction of the amine sites with  $\text{Ru}(\text{bpy})_3^{3+}$  accompanying rapid deprotonation.



The intermediate radical anion  $\text{H}_2\dot{\text{N}}\text{CRCO}_2^-$  is a strong reducing agent and produces an excited state,  $\text{Ru}(\text{bpy})_3^{2+*}$ , in an electron-transfer reaction with  $\text{Ru}(\text{bpy})_3^{3+}$ .



When the excited state decays to the ground state, the ECL is emitted with a wavelength of 610 nm.



The produced  $\text{Ru}(\text{bpy})_3^{2+}$  can be oxidized again on the platinum working electrode as in eq. (1), and the same cycle is repeated.

## 2.5 Procedure

A potentiostat/galvanostat (HA-151, Hokuto-Denko, Tokyo, Japan) was used for the operation of the valves and the generation of the ECL. To switch the valves,  $-0.9$  V was applied to the gold working electrode with respect to the Ag/AgCl electrode in the injection ports. Because of the polarity of the potential, the growth of AgCl on the Ag/AgCl electrode is promoted. Therefore, we used a two-electrode configuration to simplify the electrode structure. At the applied potential, the evolution of hydrogen gas was negligible. To generate the ECL,  $+1.1$  V was applied to the working electrode with respect to the Ag/AgCl reference electrode in the reaction chamber. After waiting 5 min for mixing, the ECL image or intensity was recorded using a cooled high-sensitivity CCD color camera (VB-7010, Keyence, Osaka, Japan) or the photodiode mentioned earlier in combination with an amplifier (C9329, Hamamatsu Photonics, Shizuoka, Japan). An electrometer (HE-104, Hokuto-Denko) was used for the measurement of the voltage signal from the amplifier. The experiment was conducted at room temperature.

## 3. Results and Discussion

### 3.1 Microfluidic transport and mixing of solutions

Figure 4 shows how the solution injected into the reaction chamber are mobilized. Here, a fluorescein solution was used. First, the two injection ports were filled with the solution. Since the bottom of the flow channels (glass) was hydrophilic, the solutions

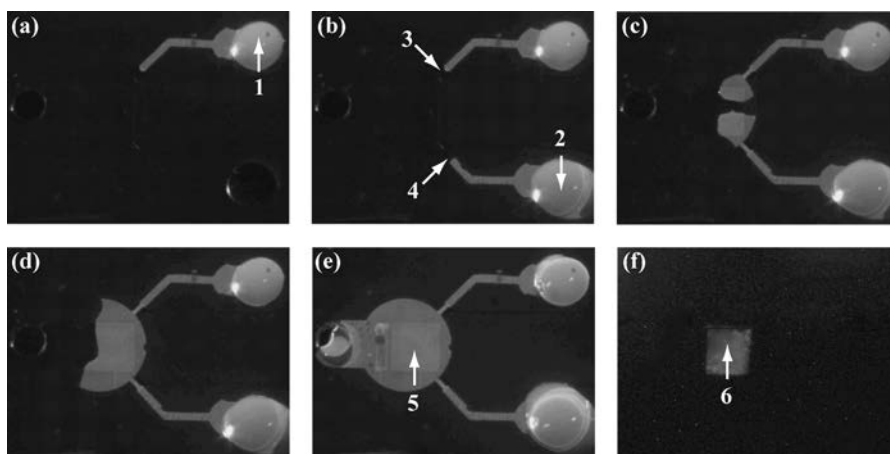


Fig. 4. Transport and mixing of solutions filled in the injection ports. 1, Injection port for the reagent solution; 2, injection port for the sample solution; 3–4, valves; 5, reaction chamber; 6, working electrode for the ECL. (f) shows the ECL observed when the reagent solution and L-proline solution (10 mM) were mixed and the working electrode for the ECL was switched on.

permeated into the respective flow channels and stopped at the edge of the valves (Figs. 4(a) and 4(b)). The flow velocity in the flow channel was approximately 5 mm/s. Although newly fabricated PDMS flow channels and electrodes provided the most effective valve action, it was gradually lost as the flow channels and the electrodes were used repeatedly. We suppose that the surfaces of the PDMS walls and the electrodes gradually became hydrophilic, possibly due to the adsorption of component molecules. As a result, the solution could not be stopped by using the valve formed in a simple straight flow channel. On the other hand, the narrowed structure at the valve area could always stop the moving solution effectively. When the valves were switched on, the surface of the electrodes became hydrophilic and the solutions passed through valve areas and permeated again into the hydrophilic glass areas of the flow channels. The solutions then spread spontaneously into the reaction chamber (Fig. 4(c)), mixed (Fig. 4(d)), and finally filled the entire reaction chamber (Fig. 4(e)).

### 3.2 Determination of amino acids

When the working electrode was switched on, red luminescence was observed almost instantly (Fig. 4(f)), indicating that the ECL reaction was proceeding. However, as seen in the time courses of the output of the photodiode (Fig. 5), the fluorescence intensity decayed rapidly and settled at a relatively stable level. The change in the output suggests that the analyzed amino acid was consumed rapidly once the series of ECL reactions proceeded in the flow channel. In the relatively stable region, the ECL was still observed. We suppose that the long-lasting low-intensity ECL was caused by the components flowing into the working electrode area from a distance including from the flow channels.

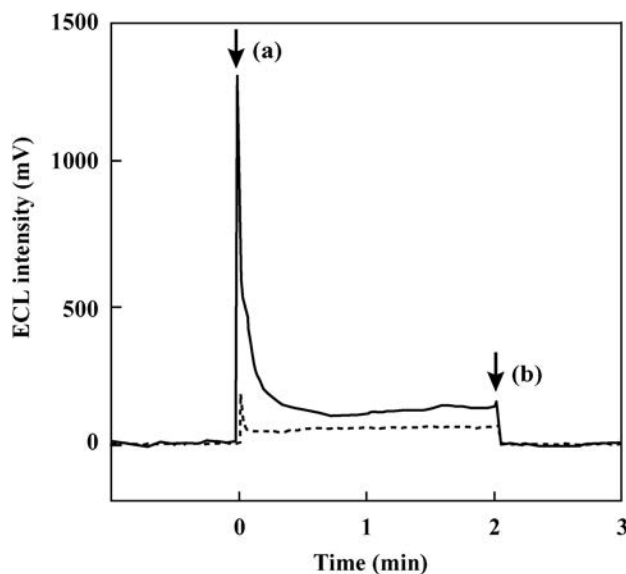


Fig. 5. Time courses of the output of the photodiode (ECL intensity) observed using L-proline solutions of 1  $\mu\text{M}$  (broken line) and 10  $\mu\text{M}$  (solid line). (a) The electrode for the ECL was switched on. (b) The electrode was switched off.

The ECL intensity depends on the pH of the sample solution.<sup>(16)</sup> To verify this, the pHs of the sample solution and the reagent solution were adjusted to a predetermined value prior to the injection and the ECL was detected. The ECL intensity increased as the pH of the solution increased up to pH 12 (Fig. 6). However, the luminescence intensity leveled off for pHs higher than 10. Although it was not significant in the present experiment, the background luminescence increases at pHs higher than 11 due to the reduction by  $\text{OH}^-$  ions. Therefore, the optimum pH for the reaction was considered to be 10 and the following experiments were conducted at this pH.

Figure 7 shows the dependence of the ECL intensity on the concentration of amino acids. Here, L-proline, L-leucine, L-lysine, L-valine, and L-histidine were used as analytes. A clear tendency was observed, and the luminescence intensity increased with increasing concentration. The concentrations of pM levels could easily be detected using the photodiode detection system. The lowest detection limit was 1.0 pM for L-proline, 5.0 pM for L-leucine and L-lysine, 10 pM for L-valine, and 1.0 nM for L-histidine ( $S/N = 3$ ). However, the scattering of the data points increased as the concentration decreased, which may actually limit the lower detection limit that can provide reliable data. The scattering of the data points may result from stray light and may be reduced by considering an appropriate covering for the device. The efficiency of reactions shown in Fig. 3 depends on the side group of the amino acids. It has been found that electron-withdrawing groups tend to decrease luminescence, whereas electron-donating group



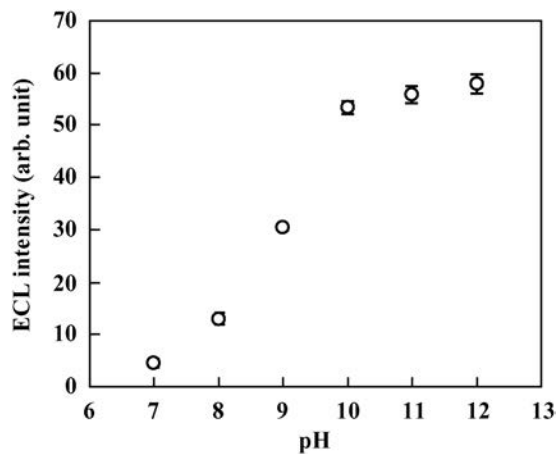


Fig. 6. Dependence of the ECL intensity on the pH of the solution. The sample solution contained 10 mM L-leucine. The error bars represent the standard deviations of the five measurements.

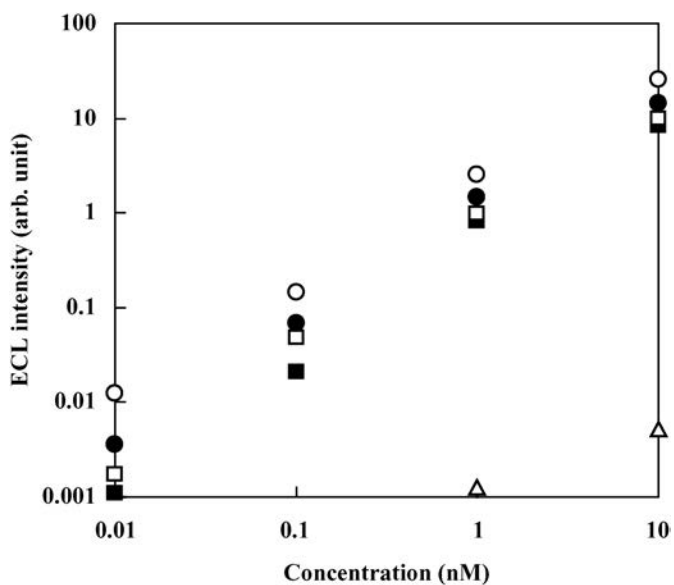


Fig. 7. Calibration plot for the device. ○, L-proline; ●, L-leucine; □, L-lysine; ■, L-valine; △, L-histidine.

such as alkyl side chain tend to enhance luminescence.<sup>(17)</sup> The observed luminescence intensity reflects the anticipated tendency. The result demonstrated that the detection of amino acids using the photodiode can be carried out with sufficient sensitivity, which also encourages us to realize a highly sensitive integrated microsystem.

In the present system, the control of the mixing ratio was a concern. However, the scattering of the obtained results at higher concentrations suggests that the 1 : 1 ratio was achieved with a fairly good precision. Also, the small scattering of the data points at higher concentrations suggests that the 5 min mixing time was sufficiently long.

In this microsystem, the reaction chamber was made relatively large to enlarge the luminescent area and to lower the detection limits. However, the mm order reaction chamber requires a few minutes for mixing, even with small molecules. Although we waited 5 min prior to switching of the working electrode for the ECL, the waiting time should be shortened to achieve high-throughput analysis. We believe that an excellent detection limit can be achieved even in a much smaller reaction chamber, by forming the photodiode in the vicinity of the working electrode for the ECL.

### 3.3 Future directions

We have succeeded in incorporating functions including microfluidic transport, mixing, and sensing based on ECL. However, there are still a few points to be improved. The first point is the accuracy of mixing. In the present device, the solutions were simply poured into the reaction chamber from the flow channels. If the surface state of the flow channels and the reaction chamber and the viscosity of the solutions are the same, the present system can achieve mixing with a fairly accurate ratio. However, mixing may not be sufficient if more accurate fluid control is required. For this purpose, another long thin valve may be placed in the reaction chamber in such a way as to separate the two solutions injected into the reaction chamber, and may be opened after the solutions have filled their respective halves of the reaction chamber.

The second point is the control of pH. As mentioned, the luminescence intensity depends on the solution pH and increases with increasing solution pH. Although the adjustment of pH was carried out prior to the experiment, it should be carried out on the same chip. To this end, a mechanism to change and fix the solution pH is necessary. We have already proposed a pH regulator that functions automatically on the basis of the electrolysis of water using a three-electrode system in an irregular manner.<sup>(18)</sup> The device is expected to be a solution to this problem.

In this device discussed in this paper, gold electrodes in the flow channels were used as valves. Since the length of the electrode is limited, the delay of the transport of solution on the electrode is not significant. However, for faster operation, even the present response time may pose a problem. Therefore, to stop the solution effectively using a short strip of the working electrode, the change in the wettability or the contact angle should be enhanced in the valve in the mixing channel. To solve this problem, an effective method is to form a microscopic three-dimensional structure under the electrode.<sup>(19,20)</sup> We have already verified that the microscopic three-dimensional structure enhances the change in wettability. Furthermore, the microscopic structure can make the change more rapid. The technique will be useful for improving the response performance.

#### 4. Conclusions

A microsystem that can conduct the injection of solutions, mixing, and sensing was constructed using valves based on electrowetting and ECL. The hydrophilic flow channels can mobilize solutions spontaneously, and the valves can control the injection of two solutions. Even with the simple design and operation, the control of the mixing ratio was fairly accurate. By applying a positive potential to the working electrode, a distinct ECL can be observed immediately. The luminescence intensity increases as the solution pH increases, and the pH should be adjusted at 10 to obtain the highest sensitivity. With the photodiode incorporated in the reaction chamber, lower detection limits on the order of lower pM levels were achieved.

#### Acknowledgments

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