Microscale Chemical and Enzyme Sensor Arrays Prepared by Microcontact Printing

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Fluorescence-based integrated chemical and enzyme sensor arrays on which two kinds of 10 µm inside diameter (i.d.) sensor spots were arranged alternately on one chip were developed using microcontact printing. Fluorescein isothiocyanate (FITC), a ruthenium complex, and europium tetracycline (EuTc) were stamped onto diamond-like carbon (DLC) sputtered glass slides for pH, oxygen, and hydrogen peroxide sensors, respectively. After printing oxygen sensor spots, pH sensor spots were stamped between the oxygen sensor spots using the same polydimethylsiloxane (PDMS) stamp. In the same way, glucose oxidase and lactate oxidase spots were stamped in an alternating pattern onto H_2O_2 sensor spots. Each sensor in the integrated sensor showed specific responses independently.

1. Introduction

Recent studies in biotechnology focus on the investigation of single cell function and cell-cell communications. Therefore chemical and biochemical imaging technologies which can visualize each cell activities *in situ* are strongly required.

Chemical imaging technologies for pH[1,2] and O_2[3] have been reported, but they required expensive and special instruments. Simpler methods which require only common tools in biochemical laboratories are desired. Therefore we focused on fluorescence-based chemical sensor technologies.

We have developed micro-arrayed chemical and enzyme sensors for parallel monitoring of single cell activity.[4] These sensors consist of an optical sensor film and a microwell array prepared with polydimethylsiloxane (PDMS). Differences in respiration can be detected at the single cell level using a microarrayed oxygen sensor.[5] By combining these microarrayed chemical sensors with the single cell collection system, single-cell-based detection and collection systems could be developed.[6]

By arranging the chemical sensor films as a million spots of 10 µm inside diameter

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(i.d.) on one chip, a novel chemical imaging device might be realized. The integration of several types of sensor spots on one chip might make possible a new bioimaging method. Microcontact printing technology[7] using PDMS stamps is an easy and simple technique for micropatterning functional chemicals. This method is similar to the normal stamping process, except the pattern of the stamp is very small. Printing of one type and multiple types of various functional molecules (alkanethiols[7]) and biomaterials (proteins,[8] peptides,[9] bacteria[10]) has been reported. Therefore we applied this technology to the preparation of a chemical imaging device. The use of microcontact printing technology for chemical sensor array preparation has the following advantages: (1) good reproducibility, (2) ease of mass production, and (3) easy integration of several kinds of sensors. Indeed, various sensor spots might be easily integrated by stamping corresponding fluorescence sensor dyes several times. In this paper, two examples of integration, a pH and oxygen sensor array, and a glucose and lactate sensor array, using microcontact printing are described.

The values of pH and oxygen concentration are good indicators for estimating cell activity. We have used fluorescein isothiocyanate (FITC) and a ruthenium complex[11] for microarrayed pH and oxygen sensors, respectively, as fluorescence sensor dyes. In the first half of this study, pH and oxygen sensor spots 10 μm in i.d., were arranged alternately on one chip using microcontact "double" printing. Enzyme sensors are known as powerful tool of *in situ* metabolome analysis at the cell and tissue level. The hydrogen peroxide (H_2O_2) sensor is the most popular and sensitive transducer for enzyme sensors. We selected europium tetracycline (EuTc),[12] the fluorescence intensity of which increases by the addition of H_2O_2, as the fluorescence sensor dye for the H_2O_2 sensor array. In the second half of this study, EuTc was stamped using microcontact printing technology and a 10 μm i.d. sensor array was prepared. In addition, either glucose oxidase or lactate oxidase was stamped onto the H_2O_2 sensor spots using the same stamp, and glucose and lactate sensor spots were arranged alternately on one chip.

2. Exprimental

2.1 *Microstamp and microcontact printer*

Microstamps were prepared with PDMS (Sylgard 184, Dow Corning, USA). The mold for the PDMS stamps was prepared with SU-8 25 (MicroChem Corp., USA) by photolithography. A PDMS stamp has an array of 10 μm i.d. spots, and their pitch is 40 μm (Fig. 1).

The ink was stamped onto a diamond-like carbon (DLC) sputtered glass slide (amine group type or SO_3 group type, Gene slide®, Toyo Kohan Co., Ltd., Japan) using a microcontact printer (PA 400, Nanotech Corporation, Japan). A microcontact printer is similar to a mask aligner, but it has two stages: one for a sample (substrate), and the other for an ink pad.

2.2 *Preparation of pH sensor array*

FITC was used as a pH sensing indicator. The fluorescence intensity of FITC increases as pH increases. FITC was covalently bound to the amine group on the surface of the amine group type Gene slide®. FITC solution (0.1 g/l) prepared with 0.05 M carbonate buffer (pH 9.5) was used as an ink.
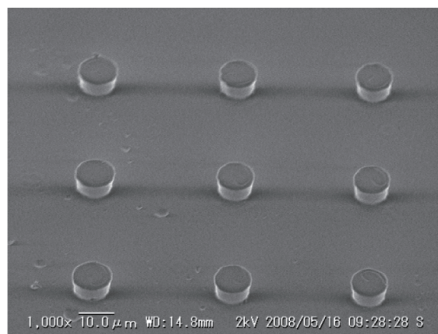
Fig. 1.   Electron micrograph of the PDMS micro stamp.

2.3   *Preparation of oxygen sensor array*

A ruthenium complex was used as an oxygen-sensitive indicator.   The fluorescence intensity of the complex increases as the oxygen concentration decreases. An oxygen sensor film was prepared by printing a mixture of 0.5 g/l dichlorotris (1,10-phenanthroline) ruthenium (II)[11] and 2.5% Nafion onto the amine group type Gene slide®.

2.4   *Preparation of hydrogen peroxide sensor array*

EuTc was used as a hydrogen peroxide-sensitive indicator.[6]   The fluorescence intensity of EuTc increases as the hydrogen peroxide concentration increases. Europium chloride (1.2 mg) and tetracycline (0.4 mg) were dissolved in 50 ml of 5 mM MOPS buffer (pH 7.0). Ink was prepared by mixing 3 ml of EuTc solution, 3 ml of 5% Nafion solution, and 1.8 mg of polyvinylalcohol. The ink was stamped onto the SO_3 group type Gene slide® using a microcontact printer.

2.5   *Preparation of enzyme sensor array*

An enzyme solution [2.5 or 10 units/ml prepared with 0.05 M phosphate buffer (pH, 7.0)] of glucose oxidase or lactate oxidase was stamped onto the H_2O_2 sensor array and crosslinked in a glutaraldehyde atmosphere for 12 h at 4 °C.

2.6   *Measurement of fluorescence intensity*

The fluorescence intensity of each spot was measured using a laser confocal high resolution microarray scanner (CRBIOIIe-FITC, Hitachi Soft Corporation, Japan) (exitation: 473 nm; emission: 535 nm for FITC and 585 nm for the ruthenium complex and EuTc). An inverted microscope equipped with a high-speed laser confocal scanner unit (CSU10, Yokogawa Electric Corporation, Japan) was also used for the fluorescence imaging of sensor arrays. Fluorescence intensity was calculated as follows.   First the "measurement area" of each sensor spot was determined using image analysis software.   The measurement area was almost the same as each sensor spot image. The averaged light intensity of all the pixels in the "measurement area" was the fluorescence intensity.

3.   Results and Discussion

3.1   *Characterization of pH sensor array*

For microcontact printing of pH sensor spots, the sensor reagent was absorbed into a lens cleaning tissue (Whatman, USA) which was used as an ink pad. The average spot size was 10.0 ± 0.5 μm ($n = 48$). Responses to 0.05 M phosphate buffer of pH 6–8 were measured. Figure 2 shows the average fluorescence intensity of 100 spots normalized with respect to the value at pH 7. Good correlation was observed between pH and fluorescence intensity.

3.2   *Characterization of oxygen sensor array*

For microcontact printing of oxygen sensor spots, the sensor reagent was dropped onto a PDMS stamp and was developed using an air gun.   The sensor response was evaluated using distilled water aerated for 30 min with oxygen, with nitrogen, and an unaerated 5% sodium sulfite solution.   Figure 3 shows the average fluorescence intensity of 99 spots normalized with respect to the value for normal distilled water. Good correlation was observed between oxygen concentration and fluorescence intensity. A fluorescence micrograph of the oxygen sensor array is shown in Fig. 4.   The average spot size was 10.2 ± 0.3 μm ($n = 25$).

3.3   *Characterization of integrated pH and oxygen sensor array*

Since the pH and oxygen sensor array was successfully prepared using microcontact printing, pH and oxygen sensor spots were arranged alternately on one chip using microcontact "double" printing. In the PDMS stamp, the distance between
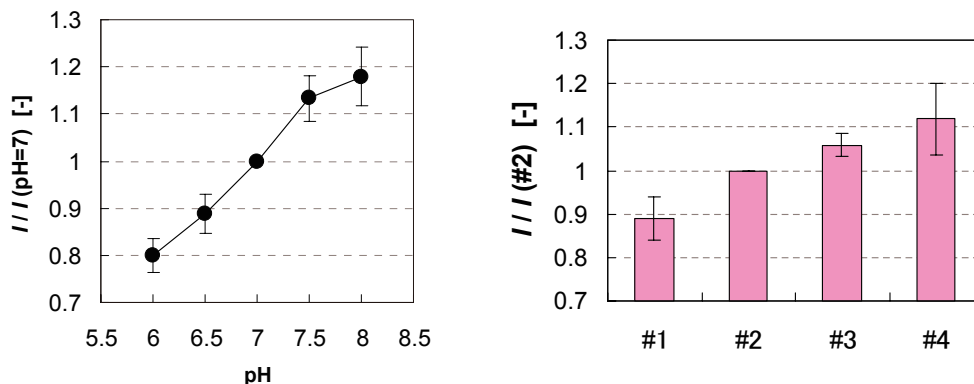

Fig. 2 (left). pH profile of the microcontact-printed pH sensor array ($n = 100$).

Fig. 3 (right). (Color online) Responses of the O_2 sensor array prepared by microcontact printing ($n = 99$). Sample solutions: #1, O_2 aerated distilled-water; #2, distilled water; #3, N_2 aerated distilled water; and #4, unaerated 5% sodium sulfite aqueous solution.
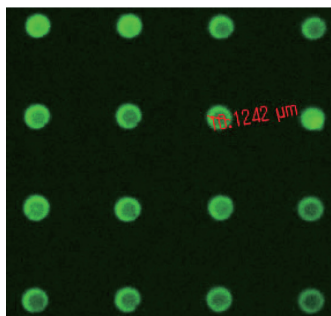
Fig. 4.   (Color online) Fluorescence micrograph of the oxygen sensor array.

two pillars was 40 μm. After the printing of oxygen sensor spots, the substrate stage was moved 20 μm, then pH sensor spots were stamped between oxygen sensor spots using the same PDMS stamp. Figure 5 shows a pseudo-colored fluorescence image of an integrated sensor array. The response of the prepared integrated pH and oxygen sensor array was measured using a microarray scanner. The results are summarized in Fig. 6. In order to cancel the effect of photobleaching of dyes, fluorescence intensity change was measured when pH was increased and decreased. Figure 6(a) shows the responses of pH sensor spots in the integrated pH and oxygen sensor array. The pH sensor spots responded to only pH changes. Figure 6(b) shows the responses of oxygen sensor spots in the integrated sensors. Oxygen sensor spots responded to only oxygen concentration changes. Although pH and oxygen sensor spots are close to each other, no interference was observed.

3.4   *Characterization of hydrogen peroxide sensor array*

Figure 7 shows the calibration curve for H_2O_2. Fluorescence intensity increased as H_2O_2 concentration increased. The RSD was 3.8% ($n = 25$). Figure 8 shows corresponding fluorescence images of the sensor array at 0 and 0.3 M H_2O_2. The EuTc solution was successfully stamped, and the average diameter of sensor spots was 10.2 ± 0.2 μm ($n = 25$).

3.5   *Characterization of the glucose and lactate sensor arrays*

Glucose oxidase was stamped onto H_2O_2 sensor spots. The average diameter of the sensor spots was 10.3 ± 0.2 μm ($n = 25$). Figure 9(a) shows a calibration curve for glucose. Fluorescence intensity increased as glucose concentration increased. The RSD was 3.2% ($n = 25$). In the same way, a lactate sensor array was prepared by stamping lactate oxidase onto H_2O_2 sensor spots. The average diameter of the sensor spots was 10.2 ± 0.3 μm ($n = 25$). Figure 9(b) shows the calibration curve for lactate. Fluorescence intensity increased as lactate concentration increased. The RSD was 2.5% ($n = 25$).
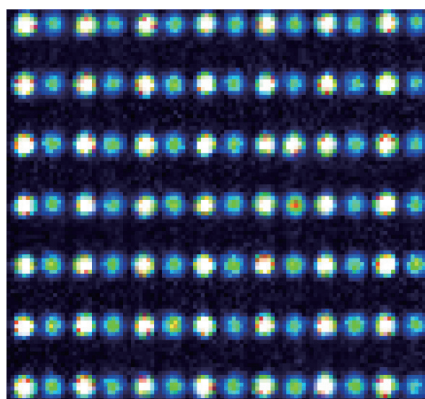
Fig. 5. (Color online) Pseudo-colored fluorescence image of the integrated pH and O_2 sensor array (exitation 473 nm; emission 535 nm). Darker spots: pH sensors; brighter spots: O_2 sensors.
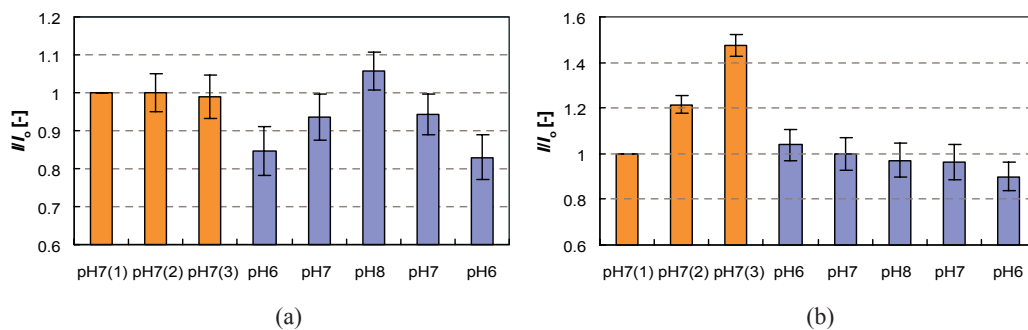

Fig. 6. (Color online) Responses of (a) pH sensor spots, and (b) O_2 sensor spots in the integrated pH and oxygen sensor array. Data for pH 7(1), pH 7(2), and pH 7(3) represent O_2 aerated, untreated, and N_2 aerated phosphate buffer (pH 7), respectively. The value of I_0 is the fluorescence intensity at pH 7(1).

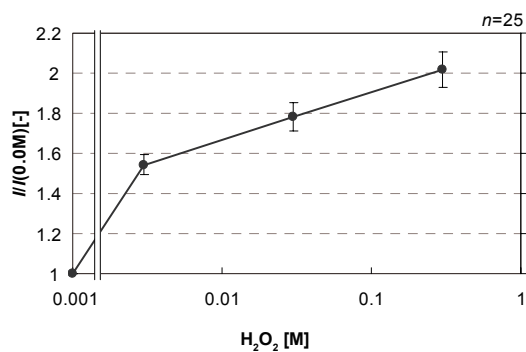Fig. 7.   Calibration curve for H_2O_2.

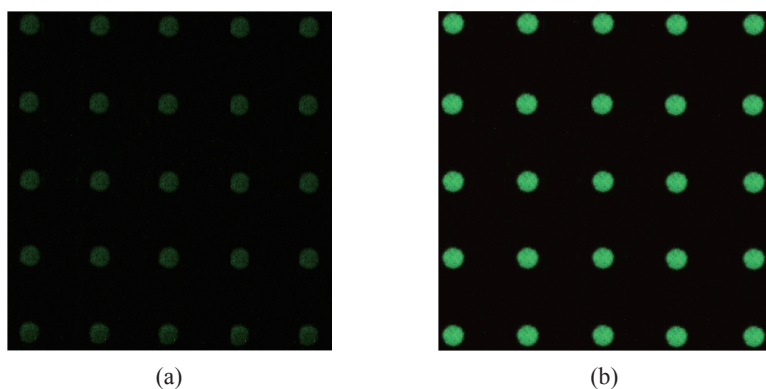(a)   (b)

Fig. 8.   (Color online) Fluorescence micrograph of the H_2O_2 sensor array (a) at 0 and (b) 0.3 M. Averaged spot intensities ($n = 25$): 0 M 2308 ± 228 (arb. unit), 0.3 M 4720 ± 491 (arb. unit).

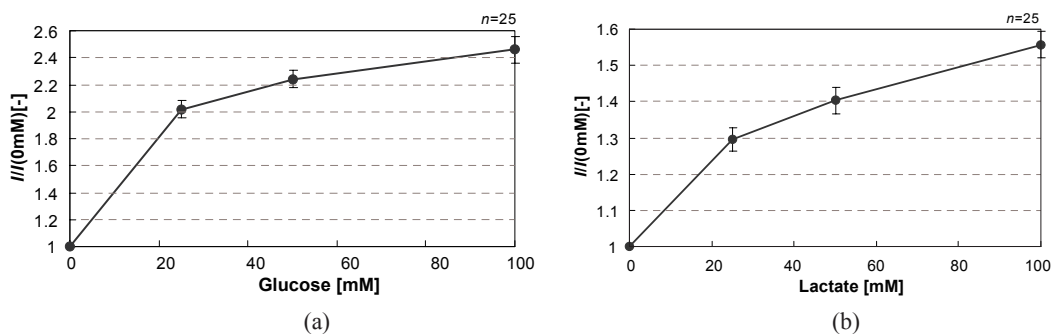(a)   (b)

Fig. 9.   Calibration curves for (a) glucose and (b) lactate.

3.6   *Characterization of the integrated glucose and lactate sensor array*

An integrated glucose and lactate sensor array was prepared by microcontact double printing. First, the H_2O_2 sensor array (spot diameter: 10 μm, spot pitch: 20 μm) was prepared by stamping EuTc ink two times 20 μm apart. Then lactate oxidase was stamped onto the H_2O_2 sensor spots and, after moving 20μm, glucose oxidase was stamped on the adjacent spots. Figure 10 shows the fluorescence image for 0 and 100 mM glucose. An increase in fluorescence intensity was observed only for glucose sensor spots.   Figure 11 shows calibration curves for (a) glucose and (b) lactate. Each sensor spot showed specific responses independently for each substrate.   These results show that this method can be applied to the integration of various enzyme sensors using oxidase enzymes. At the present stage, we have insufficient data to consider sensitivity and resolution of the chemical imaging chips, but this method might contribute to *in situ* metabolome studies of single cells or at the tissue level in the future.

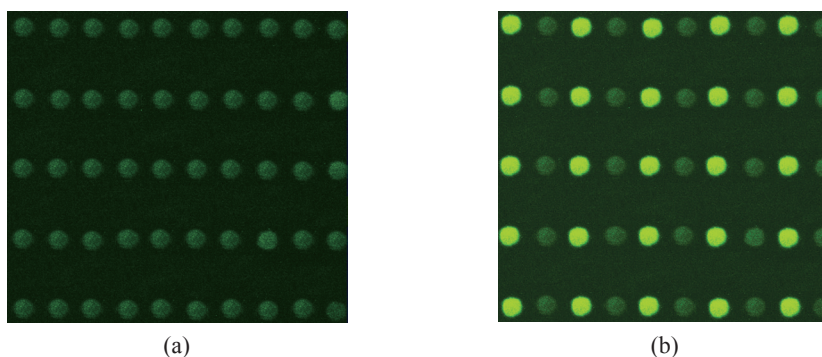(a)   (b)

Fig. 10. (Color online) Fluorescence micrograph of the integrated glucose and lactate sensor array in (a) 0 and (b) 100 mM glucose. Averaged spot intensities ($n = 25$): (a) glucose sensor: 938 $\pm$ 62 (arb. unit), lactate sensor: 766 $\pm$ 57 (arb. unit) and (b) glucose sensor: 1928 $\pm$ 127 (arb. unit), lactate sensor: 769 $\pm$ 53 (arb. unit).

(a)   (b)
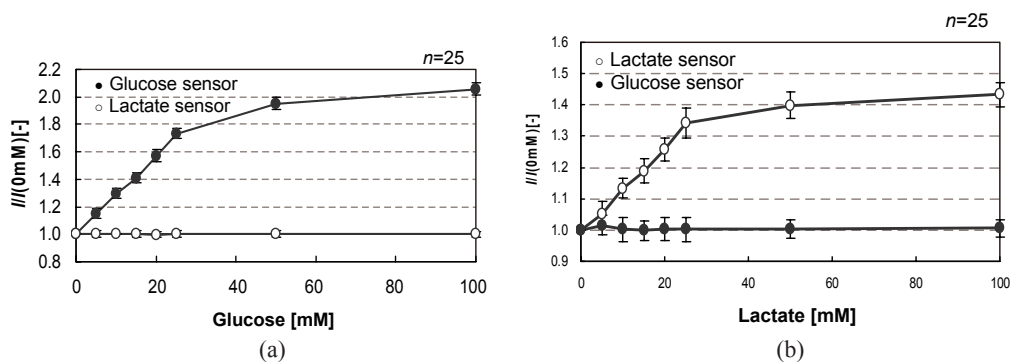
Fig. 11. Calibration curves for (a) glucose and (b) lactate on the integrated glucose and lactate sensor array.

4.   Conclusions

We developed fluorescence-based integrated chemical and enzyme sensor array chips on which two kinds of sensor spots, each 10 µm in diameter, were arranged alternately using microcontact printing. FITC, a ruthenium complex, and EuTc were stamped onto DLC sputtered glass slides for pH, oxygen, and hydrogen peroxide sensors, respectively. Different kinds of microsensor arrays could be easily integrated using microcontact "double" printing. In this paper, we presented two examples: (1) integration of different chemical sensors (pH and oxygen sensors), and (2) integration of different enzyme sensors (glucose and lactate sensors). In the case of high density integrated enzyme sensors, "crosstalk" responses caused by hydrogen peroxide produced at neighbor sensors has previously been observed.[13]   No such responses were observed in the integrated glucose and lactate sensor array in this study.

Acknowledgements

References

1   S. Inoue, T. Yoshinobu and H. Iwasaki: Sens. Actuators, B **32** (1996) 23.
2   T.Hattori, Y. Masaki, K. Atsumi, R. Kato and K. Sawada: Anal. Sci. **26** (2010) 1039.
3   H. Shiku, T. Shiraishi, H. Ohya, T. Matsue, H. Abe, H. Hoshi, M. Kobayashi: Anal. Chem. **73** (2001) 3751.
4   M. Suzuki, H. Nakabayashi, Y. Iribe and M. Honda: Micro Total Anal. Syst. 2005 **2** (2005) 1482.
5   M. Suzuki, T. Yamada, S. Kato and Y. Iribe: Micro Total Anal. Syst. 2008 **1** (2008) 438.
6   M. Suzuki, A. Murata and Y. Iribe: J. Micro-Nano Mechatron. **7** (2012) 79.
7   A. Kumar, H. A. Biebuyck and G. M. Whitesides: Langmuir **10** (1994) 1498.
8   A. Bernard, E. Delamarche, H. Schmid, B. Michel, H. R. Bosshard and H. A. Biebuyck: Langmuir **14** (1998) 2225.
9   M. Scholl, C. Sproessler, M. Denyer, M. Krause, K. Nakajima, A. Maelicke, W. Knoll and A. Offenhaeusser: J. Neurosci. Methods **104** (2000) 65.
10 D. B. Weibel, A. Lee, M. Mayer, S. F. Brady, D. Bruzewicz, J. Yang, W. R. DiLuzio, J. Clardy and G. M. Whitesides: Langmuir **21** (2005) 6436.
11 M. C. Moreno-Bondi, O. S. Wolfbeis, M. J. P. Leiner and B. P. H. Schaffar: Anal. Chem. **62** (1990) 2377.
12 M. Wu, Z. Lin and O. S. Wolfbeis: Anal. Biochem. **320** (2003) 129.
13 M. Suzuki and H. Akaguma: Sens. Actuators, B **64** (2000) 136.