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Light Addressable Potentiometric Sensor (LAPS)-Type Penicillin Sensor with Self-Assembled Monolayers and Its Image Detection

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In this study, we fabricated a light addressable potentiometric sensor (LAPS)-type penicillin sensor using the self-assembled monolayer (SAM) method and investigated its response characteristics. In addition, the application of the fabricated LAPS sensor to detect concentration distribution and speed of diffusion was also evaluated. A penicillin-sensing membrane was made using the SAM method, and immobilization was confirmed by atomic force microscophy (AFM). The enzyme response characteristics of the fabricated sensor were 60 mV/decade within the penicillin concentration range from 0.1 mM to 10 mM. The characteristics of the 2-D image resulting from the enzyme reaction between penicillin and penicillinase were also investigated. Image detection used a line scanning sampling method and required approximately 10 min for 128 × 128 pixels over a 1 × 1 cm sensing area.

1. Introduction

Since Caras introduced the idea of an enzyme field-effect transistor (ENFET),[(1)] much progressive research has been carried out on semiconductor-based biosensors that use the ability of organisms to discern active molecules. This has resulted in the production of various ENFETs,[(1)] ion selective field effect transistors (ISFETs),[(2,3)] and light addressable potentiometric sensors (LAPS).[(4–6)] The characteristics of these sensors include fast response, high accuracy, and easy of fabrica

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tion using semiconductor processes. In particular, LAPSs can detect potential changes at the interface between an electrolyte and an insulator. The technique has several advantages, including a high electrochemical stability and the ability to measure various materials by local light illumination without any additional process. LAPS systems have a wide range of analytical applications, ranging from measurements of microcells, such as pathogenic microorganisms, to macromolecules, such as biological toxins, hormones, and *E. coli* colonies.[5,7] In addition, various recent studies have used "light addressablility" to determine the two-dimensional distribution of concentration or to detect diffusion velocity, both of which appear as a result of cellular metabolism.

For example, Nakao and Yoshinobu published studies on pH image detection, where a two-dimensional pH distribution was measured using a scanning laser beam on silicon, while a high-resolution pH image sensor was used for minute tissue observation.[8–10] The image detection for the concentration distribution or diffusion velocity of the biomaterial is used extensively in fields such as fermentation monitoring, medicine, pharmacy and biochemistry.

Nonetheless, the sensitivity and stability of biosensors are affected by the method used to immobilize the sensing membrane. To increase the sensitivity of sensors, the amount of enzymes immobilized on the sensor surface must be maximized, as long as the immobilized enzymes do not hinder each other. In particular, the immobilized enzymes must maintain their bioactivity on the sensor surface during the sensor preparation and operation.

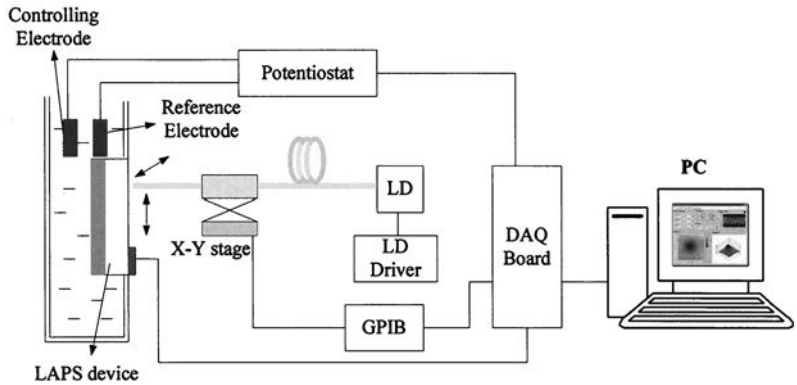
However, in the case of chemical/biosensors and LAPS images, a polymer matrix, such as a photocurable polymer,[11] PVC,[12] or gelatin,[8] is used to immobilize the sensing membrane on the sensor surface. As a result, these methods have certain disadvantages; for example, if the adhesion to the sensing surface is not adequate , the molecules may escape from the membrane in a short time. Furthermore, since the coupled molecules can attack the immobilized enzymes and the enzyme activity can be diminished during a catalytic reaction, immobilization may not really be suitable.

To solve such problems related to the immobilization of the sensing membrane, in this study, we evaluated the immobilization of a sensing membrane on a sensor surface of $Si_3N_4/SiO_2/Si$ using the self-assembled monolayer method.[13] Penicillinase was then immobilized on the sensor surface using aminoprophytrimethoxysilane (APTMS) with amine groups and glutaric acid.

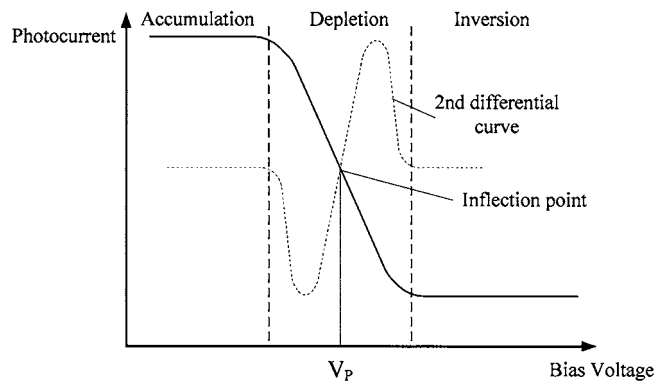
To measure the response and image characteristics of the penicillin, the measuring system was simplified to include an amplifier, an A/D converter, a filter, and an x-y stage controller for the hardware, and LabVIEW software for the display. In tests, an image based on 128 × 128 pixels over a 1 × 1 cm sensing area was obtained using the fabricated LAPS and measuring system.

2. Sensing principle of LAPS and image detection mechanism

An important structure for chemical sensors on silicon is the electrolyte /insulator/ silicon (EIS) structure.[14] Figure 1(a) shows a schematic diagram of the LAPS with an EIS structure. Alternating light is illuminated onto the LAPS device and a DC voltage is



(a)



(b)

Fig. 1. Schematic diagram of detecting system and graph of photocurrent vs bias voltage. (a) Schematic diagram of LAPS system. (b) Relationship of photocurrent vs bias voltage.

applied to the electrolyte. The DC bias voltage makes an inversion layer in the LAPS device, then the charges in the inversion and insulator layer are modulated by an electron-hole pair to make the AC photocurrent [15] (I_p) given by eq. (1)

$$I_p = I_1 \cos \omega t, \tag{1}$$

where I_1 is the amplitude of the photocurrent and ω is the frequency of the addressed light. However, if the DC bias voltage creates an accumulation state, no photocurrent is generated, as there is no electric field on the silicon surface. Therefore, the I-V curve is a function of the DC bias voltage, as seen in Fig. 1(b), which shows significant changes between an accumulation state with no photocurrent and an inversion state with the maximum photocurrent.[16] As such, the sensitivity of the LAPS to an enzyme reaction is determined by measuring the shift in the I-V curve and is expressed by the voltage (V_p) at the inflection point of the I-V curve.

Meanwhile, for a LAPS biochemical imaging sensor, the image is detected by a pixel method (PDM), where the modulated laser light is illuminated on the back side of the sensor by pixels and the photocurrent for each pixel measured at a fixed DC bias voltage.[9,17] Figure 1(a) shows a schematic diagram of the system. If modulated laser light and fixed DC bias voltage are applied to the sensor, the resulting AC photocurrent, including the light position components, is changed by the potential variation on the sensor surface due to an enzyme reaction. In this case, the ACßß photocurrent (I_{pixel} (x,y)) components include the light source frequency, the amplitude of the current, and the x-y position. Therefore, eq. (1) can be expressed as follows:

$$I_{\text{pixel}}(x, y) = I_{x,y} \bullet \cos(wt). \tag{2}$$

The visual image (Image(X,Y)) represents a 2-D distribution of the photocurrent resulting from each pixel, as given by eq. (3).

$$\text{Image}(X,Y) = \sum_{x=1}^{X} \sum_{y=1}^{Y} \left| I_{\text{pixel}}(x,y) \right| \tag{3}$$

3. Experiment

3.1 *LAPS device and measurement system*

Si (p-type, $\rho = 6 \times 12$ Ω.cm, (100)) was used as the substrate for the LAPS device. An oxide layer about 300 Å thick was grown by dry oxidation, while silicon nitride about 800 Åm thick was deposited by LPCVD. A Au electrode was formed by thermal evaporation on the back side of the LAPS device, except for the illuminated region of the light source. The measurement system consisted of a potentiostat, a light source, a reactive chamber, and a signal detector, as shown in Fig. 1(a). The modulated light source (1–10KHz) was a pigtailed laser diode of 830 nm, and the signal processing part and potentiostat consisted of National Instrument's LabVIEW. Thus, the system was simplified using software to handle complex signal processing. The detailed positioning of the light source was automatically controlled by the x-y linear moving stage in the LabVIEW program using the GPIB communication method.

Figure 2 shows a flowchart of the image detecting mechanism. The acquired data was sampled 128 times by scanning the light in the direction of the X-axis, which was then repeated by sampling 128 times in the direction of the y-axis. The system took 10 min to measure 128 × 128 pixels over a 1 × 1 cm area.

3.2 *Preparation of sensing membrane*

The penicillin sensing membrane was fabricated using the self-assembled monolayer method, and Fig. 3 shows a schematic diagram of the immobilization reaction. To clean and produce the hydroxide layer on the Si_3N_4 surface of the LAPS device, the Si_3N_4 surface was washed and treated, following the described method in a previous report.[18] The Si_3N_4 of the LAPS device was then immersed in a piranha solution (H_2SO_4:H_2O_2=7:3), rinsed with H_2O, and immersed in 0.5 M NaOH followed by 0.1 M HCl to neutralize the surface.

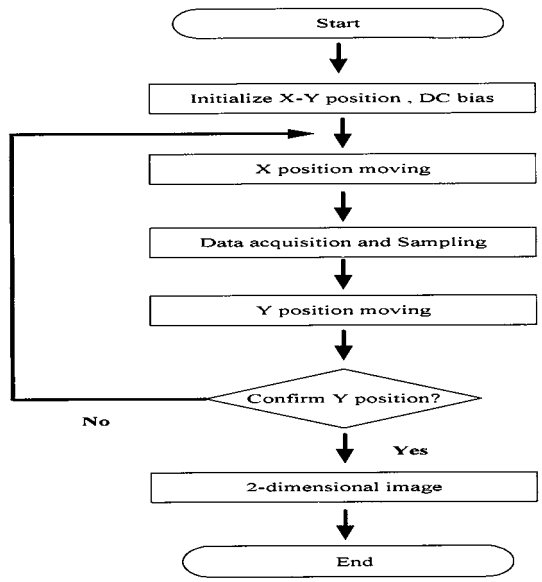


Fig. 2. Flow chart of image detection program.

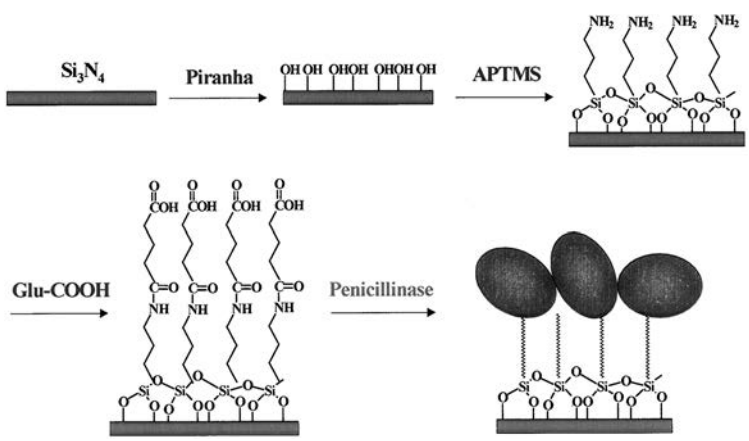


Fig. 3. Schematic diagram of penicillinase immobilization.

To immobilize the penicillinase by covalent bonding on the pretreated Si_3N_4 surface, a bifunctional crosslinker was needed to simultaneously attach to the Si_3N_4 surface and couple with the penicillinase. Thus, to make a stable coupling layer on the Si_3N_4 surface, an amine terminal silane (APTMS) was reacted with the hydroxide-layered Si_3N_4 surface using the self-assembly method. The hydroxide-capped Si_3N_4 substrate was immersed in a 2.5% (v/v) APTMS solution made using anhydrous ethanol for 30 min in a N_2 chamber, then rinsed with absolute ethanol and maintained in a heated oven at 110ºC for 10 min. As a result, the amine terminal substrate coupled with glutaric acid (GA) based on an amide reaction. The amine-terminated substrate was then immersed in a 0.3 M GA-methanol solution with 0.1 M NHS (N-hydroxysuccinimide) and 0.4 M EDC (1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydro-chloride) for 3 h. These reactions created an active surface with carboxylic acid terminal groups. For a carboxylic acid terminal substrate, the substrate was immersed in an aqueous solution with 0.1 M NHS and 0.4 M EDC for 2 h and rinsed with H_2O. The substrate was then immersed in a 0.5 mg/ml penicillinase PBS (phosphate buffer saline, pH 7.4) for 15 h.

4. Results and Discussion

4.1 *pH characteristics of LAPS device*

Figure 4 shows the photocurrent characteristic curve according to pH changes in the fabricated LAPS device. As the potential of the sensing surface changed with a variation in ion concentration, the applied DC bias voltage was also changed to maintain the same electric field in the semiconductor. Consequently, the photocurrent curve moved along the bias voltage axis, as shown in Fig. 4; the sensitivity of the fabricated LAPS device was 57 mV/pH.

4. 2 *Characteristics of response to enzyme reaction*

Although Si_3N_4 has been considered as one of the better solid substrates for biochip applications due to its excellent optical characteristics including easy adaptability to the micro-patterning process, it needs complicated detection methods such as light-addressable potentiometric detection or elaborate waveguide detection techniques for the quantitative analysis of immobilized proteins. Here, we characterized the immobilization of SAMs with APTMS, GA and penicillinase, step by step, on the Si_3N_4 surface of the LAPS device using water contact angle measurements and AFM. Table 1 shows the contact angles for each substrate. The Si_3N_4 on the LAPS device exhibited slightly hydrophobic characteristics with contact angles of 62 ± 3° for a pure water droplet. Meanwhile, the piranha-solution-treated Si_3N_4 substrate was significantly hydrophilic with a contact angle of about 16º, indicating the hydrophilic character of the hydroxyl groups formed on the Si_3N_4 substrate by the piranha treatment. Each SAM, including APTMS, GA, and penicillinase, showed a distinct contact angle. The routinely measured water contact angles of SAMs bearing -OH, -NH_2 and -COOH functionalities were in accordance with published data.[19] SAMs treated with penicillinase produced a more hydrophobic surface (51–53°) than those with -NH_2 and -COOH , which formed moderately wettable surfaces. To detect the changes in the surface morphology with each SAM, AFM images were taken to measure

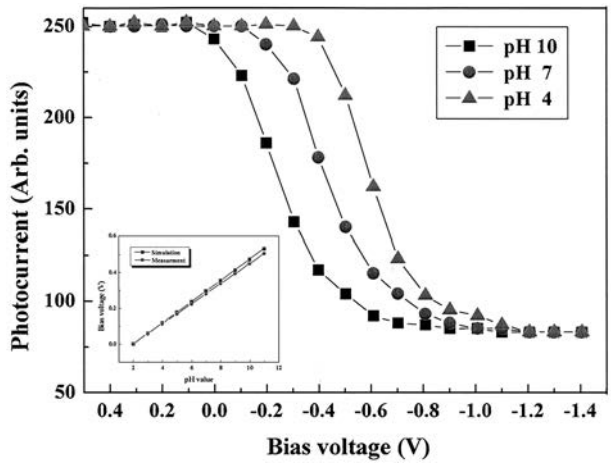


Fig. 4. Characteristic curve of pH vs photocurrent.

Table 1
Contact angle (unit: °) and RMS roughness (unit: Å) of AFM images on each SAM.

	Contact angle (unit: °)	RMS roughness (unit: Å)
Piranaha treated Si_3N_4	16.04±0.5	3.43
APTMS	41.94±1.2	6.07
GA	48.59±1.3	8.22
Penicillinase on GA	51.66±0.7	11.00

the Si_3N_4 substrate and SAMs, using the contact mode for APTMS and GA and the tapping mode for penicillinase. Figure 5 shows 1×1 μm AFM images for each SAM. The RMS roughness of each AFM image is presented in Table 1. The roughness of SAMs with -COOH showed larger values than that with $-NH_2$. This result corresponds with the published data.[19] Schöning's group[20] investigated the novel real image of penicillinase, which was immobilized on the sensor surface, by scanning electron microscopy (SEM). In the SEM picture, penicillinase had a globular shape with a diameter of 100 nm, on the average. The size of domains in this study, Fig 5(d), was almost 100 nm. Therefore, we can recognize the immobilization of penicillinase on the APTMS- and GA-modified Si_3N_4 substrate.

To detect biological activity between penicillin and penicillinase, the change in the photocurrent curve with different concentrations of penicillin G solution was measured using the penicillinase-immobilized LAPS device. The penicillin G solution was made by adding penicillin G (potassium salt, 1600 unit/mg, Aldrich) to a pH 7 Tris buffer solution. Figure 6(a) shows the photocurrent curve resulting from the enzyme reaction. The

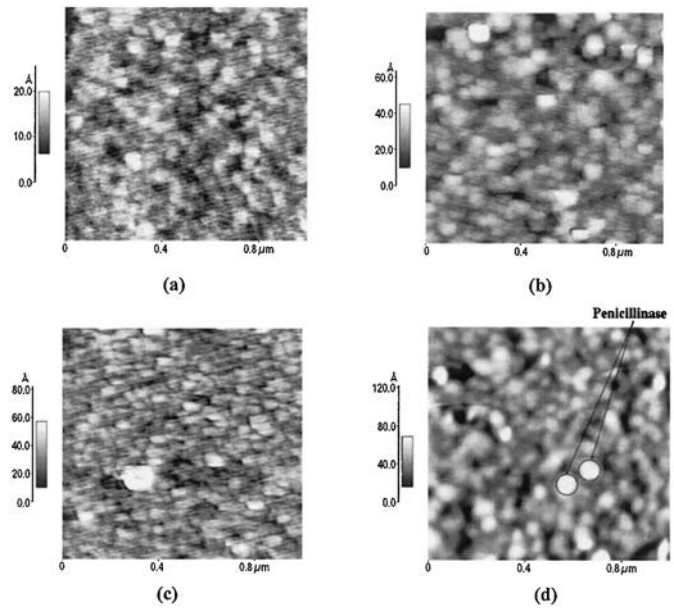


Fig. 5. AFM images for immobilizing penicillinase on Si_3N_4 thin film. (a) Piranha-treated Si_3N_4. (b) APTMS monolayer on Si_3N_4. (c) GA monolayer on APTMS. (d) Immobilized penicillinase on GA.

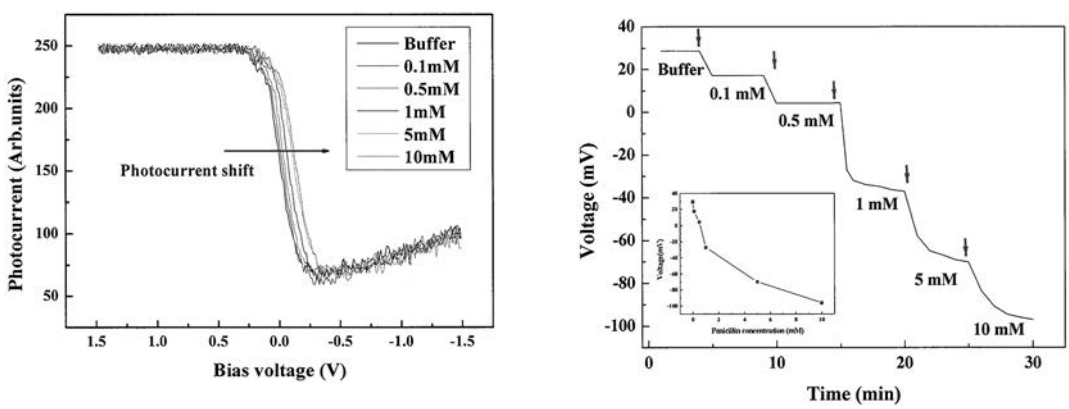
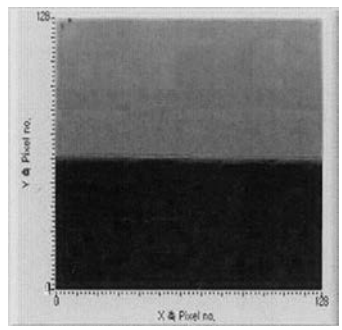


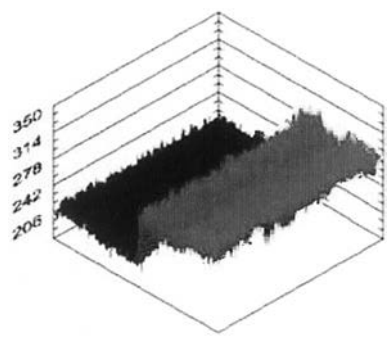
Fig. 6. (a) Response characteristics according to penicillin concentration. (b) Potential variation as function of detecting time and penicillin concentration (inset).

concentration change in the hydrogen ions†created by the enzyme reaction induced a variation in the surface potential. Therefore, the photocurrent curves shifted according to an increase in the penicillin concentration from 0.1 mM to 10 mM. Figure 6(b) shows the sensitivity of the fabricated sensor when penicillin concentration was increased from 0.1 mM to 10 mM at intervals of 5 min. The sensitivity was 60 mV/decade, which is similar to Schöning's results using a hetero-bifunctional cross-linker in 1996.[21]

In addition, to measure the biochemical image of the penicillin, a circular penicillin-sensing membrane with an area of 0.5 × 1 cm^2 was formed on the surface of the LAPS device. Figure 7(a) and 7(b) show the 2-D and 3-D images, respectively, resulting from the



(a) 2D image



(b) 3D image

Fig. 7. Image characteristics according to the penicillin and penicillinase reactions.

enzyme reaction between 10 mM of penicillin G and the immobilized penicillinase on the sensor surface. The images also correspond to the amplitude of the AC photocurrent at each pixel. The fabricated system took 10 min to measure 128 × 128 pixels over a 1 × 1 cm area.

5. Conclusions

In this study, we developed a LAPS penicillin image sensor with SAMs that can detect a 2-D distribution of a pathogenic microorganism. A penicillin-sensitive LAPS was fabricated by cross-linking penicillinase with a bifunctional crosslinker, glutaric acid (GA). The image display and signal processing were performed using LabVIEW software, while the detailed positioning of the light source was achieved using the automatic x-y linear moving stage in the Labview program via GPIB communication.

The fabricated device exhibited a sensitivity of 57 mV/pH within the pH range from 2 –11. The immobilization of the penicillin-sensing membrane was confirmed on the basis of contact angle and AFM measurements. Meanwhile, the fabricated LAPS penicillin sensor showed a sensitivity of 60 mV/decade within the range from 0.1 mM to 10 mM. Biochemical images resulting from the enzyme reaction between penicillin and penicillinase were visualized on the basis of 128 × 128 pixels over a 1 × 1 cm area, and the detection time was about 10 min. Accordingly, the proposed LAPS-type penicillin sensor is expected to have useful applications in the medical field and for the analysis of biochemical materials, along with other environmental and military uses.

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