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Temperature Alternation by an On-Chip Microheater To Reveal Enzymatic Activity of β-Galactosidase at High Temperatures

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A method to measure enzymatic activity at high temperatures by rapid temperature alternation of a microreactor with a microheater is proposed. On-chip microreactor and microheater were integrated on a glass plate by MEMS technology; this microheater can control the temperature of the microreactor with a response speed of 34.2 and 31.5 K/s for temperature rise and fall, respectively, with an accuracy of 3 °C. The enzyme, β-galactosidase, was revealed to survive short exposure (4-s pulses) to temperatures above that which would "normally" denature them. Its activity at 60 °C was revealed to be ∼4 times greater than that at room temperature. This method not only gives new kinetic information in biochemistry but also enables application in highly sensitive biosensors.

Recently, applying microelectromechanical system (MEMS) technology to chemistry, life science, and biomedical devices has been one of the major trends in these fields.[1] MEMS technology enables us to integrate sensors, actuators, electrocircuits, or probes in a small area and is widely applied to develop minute devices such as accelerometers or ink jet printers.[2] Minimizing the size of a thermal device with MEMS technology has the following advantages. The amount of heat required to control the temperature is minimized and this enables a high response speed. Sensors with high sensitivity and heaters with low energy consumption are possible because of the decline with size in heat resistance. It also enables us to integrate elements of different functions in the same area. Finally, the process is compatible with mass production. There is, however, the disadvantage that it is difficult to achieve uniform temperature distribution. Nevertheless, reducing the size of thermal devices results in a fast temperature response and offers the possibility of achieving acceleration and reduction of enzymatic activity in a short time span.[3,4]

Enzymatic activity is temperature dependent, usually showing a peak at a certain temperature, decreasing at a higher range, and then losing its activity. However, at temperatures higher than the molecular destruction temperature in a steady state, we expect the enzyme to keep active by limiting the exposure time.[5,6] Enzymatic activity at a high temperature may be measured by momentary heating. Enzymatic activity measurement at a high temperature may not only give new scientific information but also enable realization of high-sensitivity biosensors. However, to elevate and deduce the temperature in certain enzymes could not be realized by conventional technology in biochemical experiments. There are two problems to solve. One is to change the temperature rapidly, and the other is to trap certain numbers of molecules in the same place.

We have developed a microthermal device with integrated microheater and microthermosensor on a glass plate for controlling the activity of an enzyme.[3,4] This device enables rapid temperature changes under the microscope without any disadvantages such as defocusing due to thermal drift, thanks to the small amount of heat required for altering the temperature. In this report, we developed a microchamber to enclose enzymes in a temperature-controlled area and measured the chemical activity of the enzymes while inducing the heat pulses. This method enabled the activity measurement at high temperatures.

Characteristics of β-Galactosidase vis-à-vis the Temperature. β-Galactosidase (β-gal) is widely used as a reporter in molecular biology.[7] β-gal is the enzyme that catalyzes hydrolysis of fluorescein di-β-D-galactopyranoside (FDG). A buffer solution containing a fluorogenic substrate of β-gal, FDG, is hydrolyzed to fluorescein. The enzymatic reaction could take two possible pathways.[8] The first pathway consists of a two-step hydrolysis of FDG. β-Gal produces fluorescein mono-β-D-galactopyranoside (FMG). After accumulation, FMG starts to compete with FDG for the active site in β-gal, forming fluorescein. In the second pathway, FDG describes a direct transformation into fluorescein. Fluorescent intensity of both FMG and fluorescein is detected as

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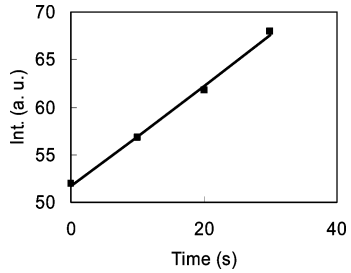

Figure 1. Time course of the fluorescent intensity of the product produced by the activity of β-gal. The intensity increases linearly when enough FDG exists. The fluorescent intensity represents the amount of the product produced by β-gal. We can measure an activity of β-gal from the gradient of the time course of the fluorescent intensity with an accuracy better than 0.8%.

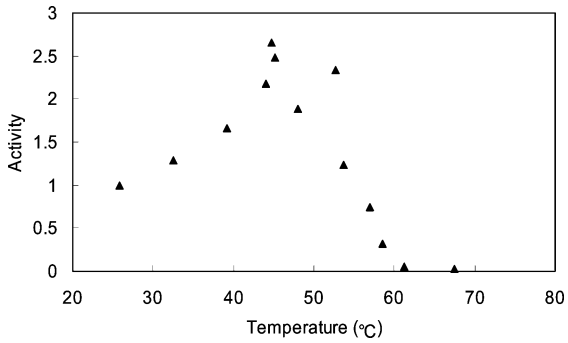

Figure 2. Activity of β-gal vis-à-vis the temperature measured by bulk experiment. It shows higher activity at a temperature higher than room temperature and shows a peak at ~45−50 °C, a decrease with temperature after the peak, and complete cessation of activity over 60 °C. Activity was determined by the rate of increase of the fluorescent intensity over a period of 2 min and standardized by the intensity at 25.8 °C.

total output.[9] Fluorescent intensity represents the amount of the product produced by β-gal.[10] Therefore, we can measure the activity of β-gal from the time course of the fluorescent intensity (Figure 1). Activity of β-gal vis-à-vis temperature measured by bulk experiment is shown in Figure 2. It shows higher activity at higher than room temperature and shows a peak at ~45−50 °C, a decrease with temperature after the peak, and complete cessation of activity over 60 °C. Activity was determined by the rate of increase of the fluorescent intensity over a period of 2 min and standardized by the intensity at 25.8 °C. The sample was returned to room temperature every time during exposure of the emission light to eliminate the effect of the fluorescent intensity change dependent on the temperature.

Temperature-Controlling Microdevice. A microheater that can rapidly alter temperature and a microthermoresistive sensor were integrated on a glass plate by patterning nickel on the glass plate (Figure 3b). Ni is used for both the heater and the sensor for the sake of simplicity in the fabrication process since the device should be compatible with mass production. First, we rinse the glass plate (24 × 36 mm^2; Matsunami) by immersing it in 20 M KOH solution for more than 8 h. Next, we evaporate 50−600-nm-thick Ni onto the glass plate with a vacuum evaporator (Quick

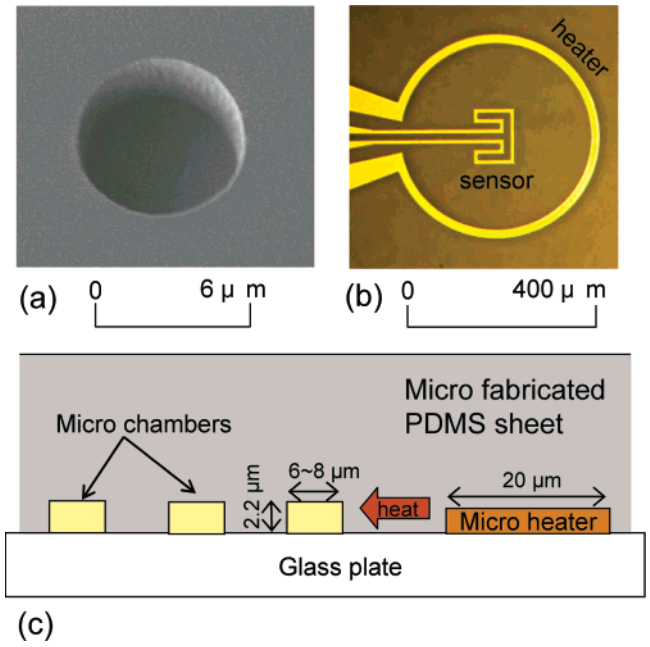

Figure 3. (a) SEM image of patterned PDMS sheet. The 56.0-fL chambers had STD of 1.63 in volume. (b) Optical microscope view of integrated microheater and microthermosensor on a glass plate. (c) Schematics of the temperature-controlled microchamber array.

Mini Vacuum System SVC-700 Turbo-TM Sanyu and Heating Power Supply SVC-700-2 Sanyu), spin-coated photoresist (S1818: Shipley Far East), and patterned by photolithography. Ni etching was done by Al etchants (Wako, Osaka, Japan). The Ni-patterned glass plate was placed onto a pitch conversion board to connect it to the lead wires by wire bonding. The temperature was changed by the heater and detected by the thermosensor. The microheater raises the temperature by applying heat by Joule heating. The resistance of the thermosensor was measured and calibrated in an aqueous environment.

The chip was also equipped with a microreaction chamber array that trapped enzymes in the temperature-controlled area (Figure 3a). Poly(dimethylsiloxane) (PDMS) was used to form the microchamber because the characteristics of PDMS have the following advantages:[11] It has no affinity for biomaterials such as proteins and cells.[12] It can be easily patterned into minute structures. It is transparent and easy to observe the target inside the structured PDMS. It is compatible with mass productivity. Its adhesion to the flat surface is strong, and it is easy to seal with flat substrate. The shape of the chamber is cylindrical with a height of 1.5−2.2 μm, a diameter of 1−8 μm, and a volume of 1−˜110 fL. This enables measurement of the enzymatic activity of a very small number of molecules, even a single enzyme,[10,13] by restricting the diffusion of products. The process is as follows (Figure 4): First we spin-coated the photoresist (S1818) on the top layer of the silicon on insulator wafer and patterned by photolithography followed by deep-RIE etching (STS), which was stopped by the SiO_2 layer. After removing the photoresist with acetone, the patterned wafer was treated with CHF_3 plasma to

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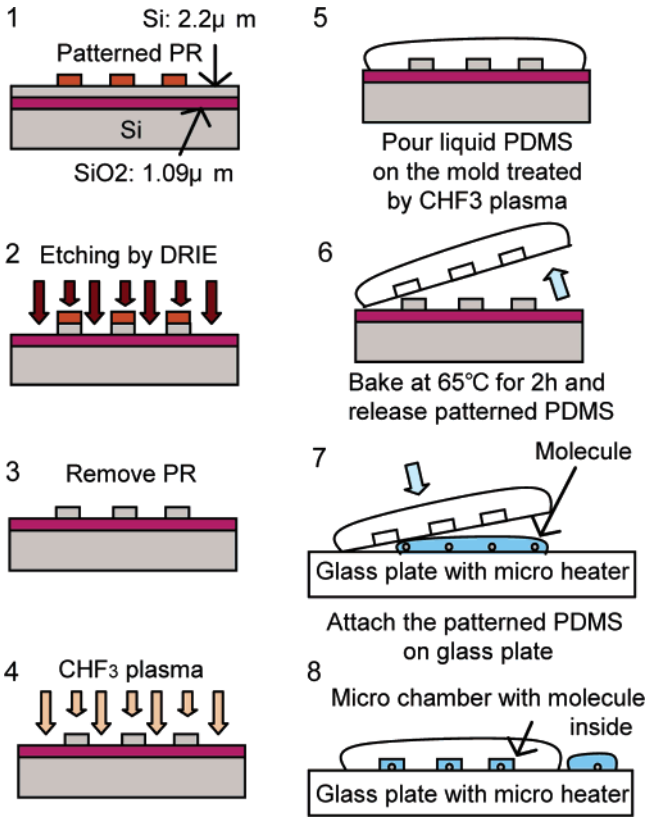

Figure 4. Process of microchamber array and process to confine enzyme in the microchambers. The solution with enzyme was poured on the temperature-controlled glass plate, and a patterned PDMS sheet was placed on top to enclose the solution in the microchamber.

attenuate the adhesion between the wafer and PDMS sheet. Liquid PDMS was poured onto the wafer and peeled off after it became solid on the hot plate (60 °C, 2 h). The temperature-controllable microchamber array was produced by attaching patterned PDMS sheets on a Ni-patterned glass plate (Figure 3).

The temperature response speed of the device was measured by inducing step input to the microheater and proved to be 34.2 and 31.5 K/s for temperature rise and fall, respectively. The accuracy of the thermosensor was 1.0 ± 0.7 °C. The temperature distribution around the microheater was measured by fluorescent dye solution. The fluorescent intensity of rhodamine B (Wako; 183-00122, 100 μM) solution depends on the temperature with high reversibility. A temperature distribution in the local area was calculated from the distribution of the fluorescent intensity of the rhodamine B solution penetrated into the PDMS sheet by sandwiching between glass plates. The temperature distribution around the microheater was successfully calculated from the intensity distribution. Panels a−d in Figure 5 show the microscopic view when the thermosensor is at 75.0, 67.8, 57.1, and 42.0 °C, respectively. Panel e shows the cross-sectional temperature distribution calculated from the fluorescent intensity. When the temperature in the center of circular heater was 75.0 °C, the temperature difference inside the circular heater was smaller than 2 °C. When the absolute temperature was lowered, the temperature difference inside the circular microheater became smaller. This indicates that all the microchambers inside the circular microheater have a temperature difference of less than 2 °C between the integrated microthermal sensor. All the enzymatic activity measurements were performed inside the circular microheater. Therefore, with this temperature distribution measurement, together with the accuracy of the thermosensor itself, we can determine the local temperature of the microchamber with an accuracy of 3 °C.

EXPERIMENTAL SECTION

A 37 nM concentration of β-gal solution was mixed with 400 μM FDG in 100 mM phosphate buffer, pH 7.5 (in the presence of 0.05−10 mg/mL BSA, 1 mM $MgCl_2$, and 2 μL/mL mercaptoethanol). The mixed solution was poured onto the Ni-patterned glass plate, and the PDMS sheet was placed on top to enclose the solution in the microchambers. Since PDMS is transparent, we can observe the fluorescent intensity inside the microchamber with an optical microscope (Figure 6). To avoid the fluorescent intensity quenching problem, the exposure time was limited to 1 s. The images were taken by a CCD camera (Hamamatsu)

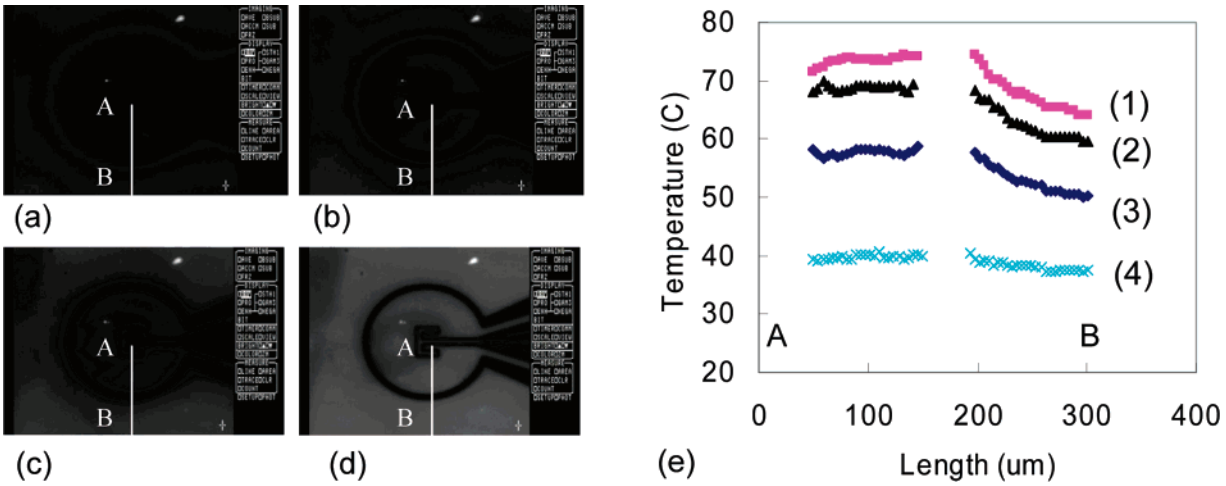

Figure 5. (a)−(d) Microscope view when the thermosensor shows 75.0, 67.8, 57.1 , and 42.0 °C, respectively. (e) Cross-sectional temperature gradients. *X* axis represents the distance from the point A shown in (a)−(d). The area from 0 to 40 μm and ~170 μm was subtracted since the micropatterned heater and sensor do not transmit fluorescent light. The data show the gradient when the thermosensor shows 75.0 (1), 67.8 (2), 57.1 (3), and 42.0 °C (4), respectively. This indicates that all the microchambers inside the circular microheater have a temperature difference of less than 2 °C between the integrated microthermosensor. With this temperature distribution measurement, together with the accuracy of the thermosensor itself, we can determine the local temperature of the microchamber with an accuracy of 3 °C.

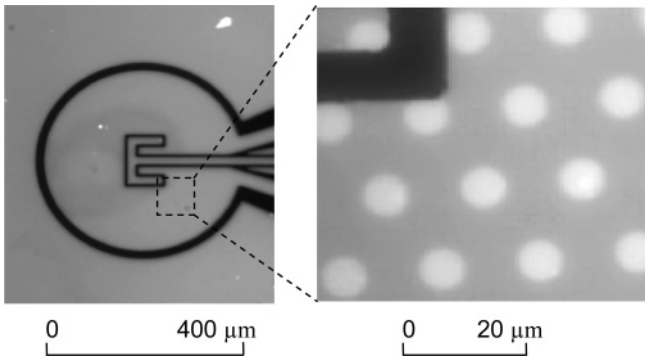

Figure 6. Microscope view of fluorescent intensity of the products produced by the enzymatic activity of β-gal inside the microchambers. The black area is the microthermosensor.

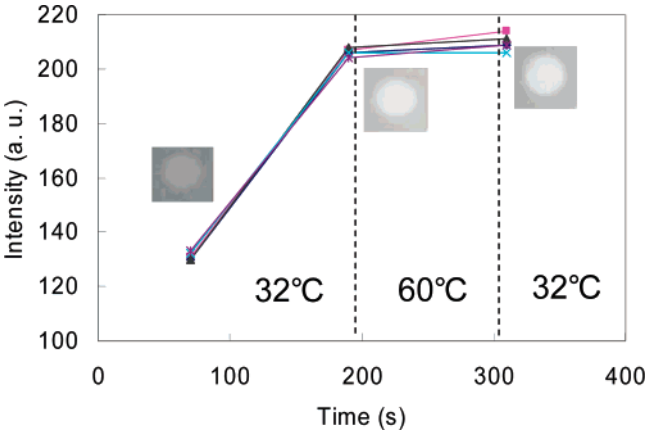

Figure 7. Intensity−time curve at temperature from 32 to 60 °C and back to 32 °C. The fluorescent intensity did not increase after raising the temperature to 60 °C. The lines follow the intensities of four individual microchambers. This indicates that the enzyme had been damaged and ceased its activity at 60 °C within 2 min.

through the optical microscope (IX70, Olympus) and recorded on a digital videocassette (Sony). The fluorescent intensity was measured by the software ScionImage. In this experiment, the volume of the microchamber was 60 fL; at a β-gal concentration of 37 nM, each chamber contained ~1300 enzymes.

RESULTS AND DISCUSSION

When the temperature of the enzyme contained in the microchamber was increased from 32 to 60 °C and kept for 2 min, the fluorescent intensity did not increase (Figure 7). This indicates that the enzyme had been damaged and ceased its activity at the high temperature, 60 °C, within 2 min. This result is also comparable with the bulk experiment in Figure 2, which shows the enzyme is inactive after exposure at 60 °C for 2 min.

Because the enzyme is expected to keep active during brief exposure even at a high temperature, we applied pulse heat to refrain from damaging the enzyme and thereby control the activity. Applying 60 °C heat pulses of 4 s, the activity is successfully accelerated. This indicates that the enzymes remain active and increase their activity over a short period, even at temperatures that cause damage after prolonged exposure. With a duty ratio of 1/3 (4 pulses in 48 s), the increased rate of fluorescent intensity changed from 0.28 to 0.59 and the increasing rate is 2.0 (Figure 8). With a duty ratio of 1/12 (1 pulse in 48 s), the ratio was 1.27. The rate of the increasing ratio (1.00:0.27) is consistent with the

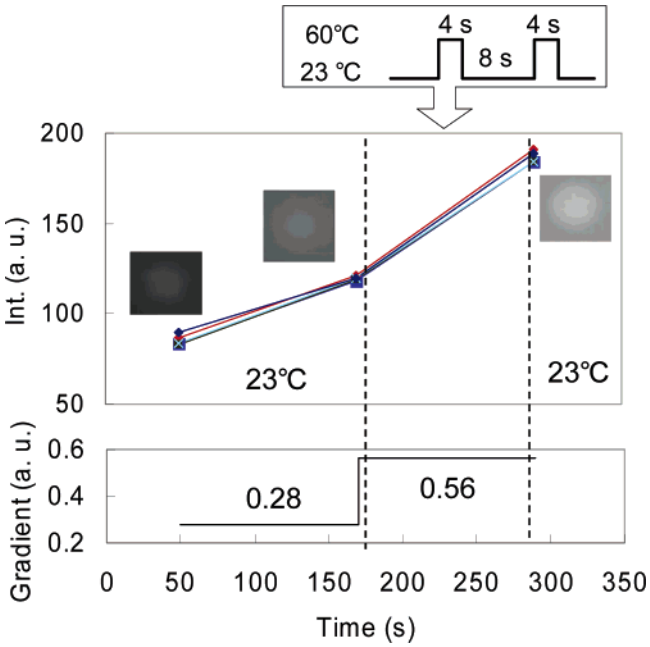

Figure 8. Activity controlled by temperature pulses with a duty ratio of 1/3 (4 pulses in 48 s). The increase rate of the fluorescent intensity changed from 0.28 to 0.56. The lines follow the intensities of four individual microchambers. Activity is successfully accelerated, and this indicates that the enzymes remain active and increase their activity over a short period, even at temperatures that cause damage after prolonged exposure.

rate of the duty ratio (1/3:1/12). This shows that the activity of the enzyme was controlled by the heat pulses. Additionally, the increase ratio of activity was also controllable by changing the duty ratio of the heating pulses.

Enzymatic activity can be calculated from the following formula:

$$\frac{4V_{60} + 44V_{23}}{48V_{23}} = 1.27 \leftrightarrow \frac{V_{60}}{V_{23}} = 4.2 \qquad (1)$$

and

$$\frac{4V_{60} + 8V_{23}}{12V_{23}} = 2.0 \leftrightarrow \frac{V_{60}}{V_{23}} = 4.0 \qquad (2)$$

where V_{60} is the fluorescent increase rate at 60 °C and V_{23} is that at 23 °C. The proximity between 4.2 and 4.0 reinforces confidence that the activity is increased by the heat pulses. From the equations, the activity of β-gal at 60 °C is ~4 times higher than at 23 °C. These are the kinds of results of the new experimental method to measure enzymatic activity at high temperatures that could not be achieved without the rapid temperature switching ability of the device.

CONCLUSIONS

Temperature control with a response speed of 34.2 and 31.5 K/s for temperature rise and fall, respectively, in a small reaction chamber (1−110 fL in volume) was achieved by MEMS technology; this enabled inducing heat pulses in the microchamber containing enzymes. The temperature accuracy was evaluated to

be 3 °C by measuring the distribution around the microheater with rhodamine B solution.

Enzymatic activity measurement at a temperature higher than the molecular destruction temperature in steady state was realized by inducing heat pulses to the enzymes contained in the microchamber. β-Gal was found to keep active even at 60 °C during the short period (4 s). The activity of β-gal at 60 °C was found to be ~4 times larger than that at room temperature. Furthermore, the acceleration degree of the activity can be controlled by changing the frequency of the heat pulses. These are the results that could not be achieved without the MEMS device.

This method gives new kinetic information such as temperature stability of biomolecules and also makes it possible to perform highly sensitive experiments in biochemistry, for example, the Arrhienius plot at higher temperatures, which does not include the effect of denatured enzymes. Furthermore, this method is suitable in realizing highly sensitive biosensors. This device can be also applied to other proteins, cells, and biomaterials for respective heating experiments.

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