Imaging of Diaphorase Micropatterned at Gold Arrays with Scanning Electrochemical Microscopy

Tomoyuki Yasukawa, Norihiro Kanaya, Daniel Mandler,† and Tomokazu Matsue*
Department of Biomolecular Engineering, Graduate School of Engineering, Tohoku University, Sendai 980-8579
†*Department of Inorganic and Analytical Chemistry, The Hebrew University of Jerusalem, Jerusalem 91904, Israel*

(Received January 27, 2000; CL-000087)

Diaphorase was hydrophobically immobilized onto a self-assembled monolayer of alkanethiol at an interdigitated Au microarray. The diaphorase activity at the array substrates was imaged with the scanning electrochemical microscopy (SECM) in the presence of NADH and ferrocenemethanol as a redox mediator. The images demonstrate that diaphorase was immobilized onto the SAM layer to form a line-and-space micropattern which reflected the pattern of the Au microarray.

Miniaturization and integration of biosensors based on immobilized enzyme have been a matter of a great interest since such sensors afford sophisticated information on a minimized volume of samples. One of the key issues to develop such biosensors is micropatterning of solid substrates with enzymes.[1-4] Photolithography, currently the most popular technique for microfabrication, is not suitable for direct patterning with enzymes since the photoresists, solvents, and atmosphere employed in the processes usually damage the enzymes. Although attempts of photolithographic microfabrication to meet the requirements for enzymes have been reported, there is a need to develop novel methods for the microfabrication.

In this paper, we report micropatterning with diaphorase, a falvin enzyme, using a self-assembled monolayer (SAM) of alkanethiol at an interdigitated Au microarray electrode. Imaging of catalytic activity of diaphorase with scanning electrochemical microscopy (SECM)[5-7] demonstrated that diaphorase was immobilized onto the SAM layer by hydrophobic interaction, thereby, forming a micropattern which reflected the Au microarray. Since the formation of Au micropatterns at solid supports has been well-established, the present method using thiol SAMs is a convenient way to fabricate micropatterns with enzymes.[8]

Diaphorase purified from Bacillus Stearothermophilus (EC 1.6.99.-) was donated by Unitika Ltd. (Kyoto). Reduced nicotinamide adenine dinucleotide (NADH), *n*-octanethiol, dodecanethiol, octadecanethiol were purchased from Wako Chemicals Ltd. (Osaka) and used as received. Ferrocenemethanol (FMA) was synthesized by reducing ferrocenecarboxyaldehyde (Aldrich) with $NaBH_4$ and recrystallized twice from *n*-hexane. An interdigitated Au microarray electrode was fabricated by conventional photolithography on clean glass substrate. The microarray electrode consisted of two comb-type arrays with 4 microband elements placed in alternate rows. Each Au element was 0.25 mm long and 20 μm wide, separated 20 μm from adjacent elements. The array electrode was dipped into an ethanol solution containing *n*-alkanethiol (10 μM) for 1 h to form SAMs at the Au elements, followed by rinsing with ethanol. The substrate was then immersed into a solution containing 0.1 mM diaphorase and 0.1 M Na_2HPO_4 for 0.5 h and washed with water. The probe for SECM measurements was a Pt midrodisk electrode sealed in a glass capillary (Pt radius, 3.3 μm; tip radius, 10 μm). The fabrication procedure was reported previously.[9-11] All the measurements were performed using a self-made SECM system[2] with an Ag/AgCl (saturated KCl) electrode as a counter/reference electrode. The current was amplified with a Keithley Model 428 amplifier. For SECM imaging, the substrate was dipped in a 0.50 mM FMA solution and the probe was moved using a motor-driven XYZ stage (AME-15, Chuo Precision Co.) above the substrate at a speed of 9.8 μm/s in a constant height mode (probe-substrate distance, 5 μm). The distance was determined from the approach curves of the oxidation current for FMA. The probe potential was set at 0.40 V vs. Ag/AgCl to detect localized oxidation of FMA. The time required to obtain an image of 100 x 60 μm was 2.5 min (pixel size, 5 x 5 μm). All the measurements were performed at 25 °C in a shield box. The solutions used in the present study were prepared from distilled and deionized water from Aquarius GS-200 (Advantec) and Milli-Q Jr. (Millipore).

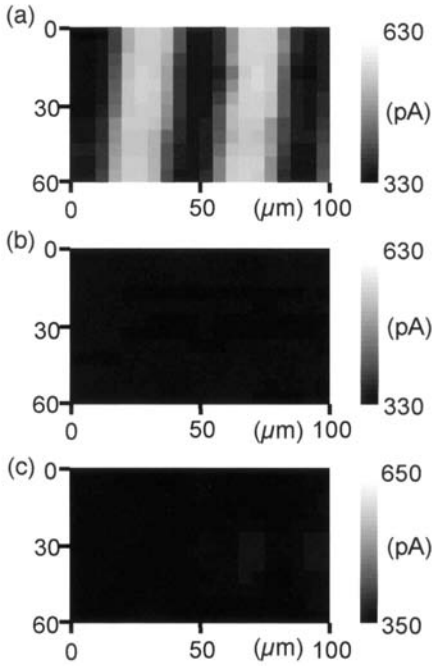

Figure 1. SECM images of microarray electrodes with (a) n-octanethiol, (b) n-dodecanethiol, (c) n-octadecanethiol SAM based on the oxidation current for ferrocenemethanol.

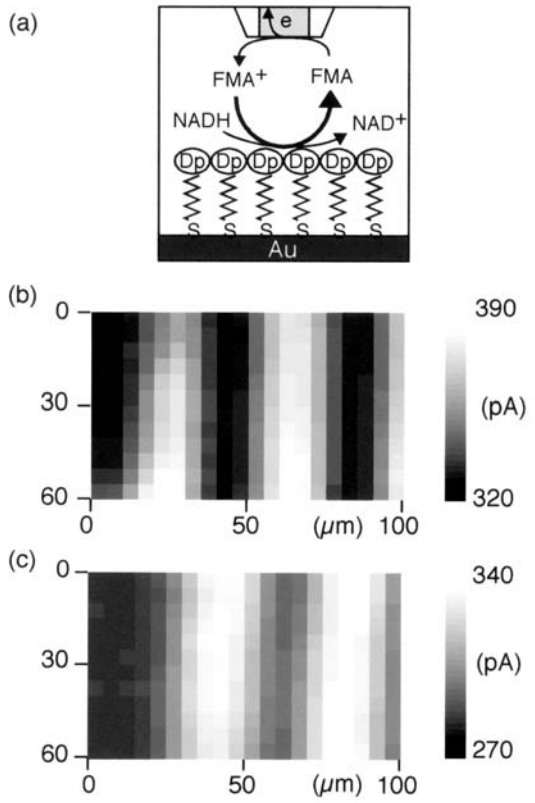

Figure 2. (a) Schematic drawing of SECM imaging of immobilized diaphorase by detection of the reproduced FMA with the microelectrode. SECM images of substrates with n-dodecanethiol (b) and n-octadecanethiol (c) SAM/diaphorase in a presence of 5.0 mM NADH.

Figure 1 depicts the SECM images of the array electrodes with three different SAMs based on oxidation current of FMA. The substrate with *n*-octanethiol SAM shows a line-and-space pattern which reflects the pattern of the Au array. This result indicates that the *n*-octanethiol SAM at the Au array does not block effectively the permeation of the oxidized form of FMA (FMA^+) into the SAM layer, probably because the alkyl chain is relatively short. FMA^+ formed at the microelectrode probe permeates and diffuses onto the Au arrays to regenerate FMA. This redox cycling of FMA/FMA^+ between the probe and Au array increases the response at the probe, resulting in appearance of the line-and-space pattern. Electron tunneling through the relatively short alkyl chain might also contribute the redox cycling. No clear image is, however, observed for the substrate with *n*-dodecanethiol or *n*-octadecanethiol SAM. The SAMs of *n*-dodecanethiol and *n*-octadecanethiol are dense and act as hydrophobic barriers to block FMA^+.

Since diaphorase tends to adsorb onto hydrophobic surfaces, the substrates with *n*-dodecanethiol or *n*-octadecanethiol SAM were dipped into a diaphorase solution. The resultant substrates showed no clear pattern in their SECM images in the FMA solution. However, the addition of NADH to the measurement solution drastically changes the SECM images (Figure 2). The images in the presence of 5.0 mM NADH in solution show clear patterns based on catalytic activity of diaphorase immobilized at the array electrodes. The lines with high oxidation current for FMA in the images coincide with the Au elements in the substrate. The line-and-space pattern is quite similar to that in Figure 1 (a) but the origin of appearance of the pattern is different. Diaphorase is adsorbed on the *n*-dodecanethiol and *n*-octadecane SAMs and catalyzes the oxidation of NADH by FMA^+ at the localized areas to regenerate FMA which can be oxidized again at the probe of SECM. This redox cycling between the probe and the immobilized diaphorase increases the response at the probe.

In conclusion, *n*-dodecanethiol and *n*-octadecane SAMs at the Au microarrays block FMA^+ from permeating into the SAMs and, therefore, block the electroreduction of FMA^+ at the Au. The SAMs adsorbs diaphorase by hydrophobic interaction. The SECM imaging of the catalytic reaction of localized diaphorase indicates that the adsorbed diaphorase forms a line-and-space micropattern which coincided with the pattern of the Au arrays. Since Au micropatterns can be fabricated easily using conventional techniques, the present procedure for microfabrication of enzyme micropatterns can be used for integration of enzyme devices.

This work was partly supported by a Grant-in-Aid for Scientific Research on Priority Area of Electrochemistry of Orderd Interfaces (09237106) from the Ministry of Education, Science, Sports and Culture, Japan. T. Y. acknowledges a research fellowship from the Japan Society for the Promotion of Science.

References

1 H. Shiku, T. Takeda, H. Yamada, T. Matsue, and I. Uchida, *Anal. Chem.*, **67,** 312 (1995).
2 H. Shiku, I. Uchida, and T. Matsue, *Langmuir*, **13,** 7239 (1997).
3 T. Wilhelm, G. Wittstock, and R. Szargan, *Fresenius' J. Anal. Chem.*, **365,** 163 (1999).
4 G. Wittstock, R. Hesse, and W. Schuhmann, *Electroanalysis*, **9,** 746 (1997).
5 R. C. Engstrom, M. Weber, D. J. Wunder, R. Burgess, and S. Winquist, *Anal. Chem.*, **58,** 844 (1986).
6 A. J. Bard, F.-R. F. Fan, J. Kwak, and O. Lev, *Anal. Chem.*, **61,** 132 (1989).
7 A. J. Bard, F.-R. F. Fan, D. T. Pierce, P. R. Unwin, D. O. Wipf, and F. Zhou, *Science*, **254,** 68 (1991).
8 G. Wittstock, and W. Schuhmann, *Anal. Chem.*, **69,** 5059 (1997).
9 T. Matsue, S. Koike, T. Abe, T. Itabashi, and I. Uchida, *Biochim. Biophys. Acta.*, **1101,** 69 (1992).
10 T. Yasukawa, I. Uchida, and T. Matsue, *Biochim. Biophys. Acta.*, **1369,** 152 (1998).
11 R. M. Wightman, *Anal. Chem.*, **50,** 1125A (1981).