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Protein Patterning Based on Electrochemical Activation of Bioinactive Surfaces with Hydroquinone-Caged Biotin

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An efficient attachment and patterning method of proteins on surfaces is crucial to the construction of protein chips[1] and bioelectronic devices.[2] In the attachment step, the site-specific immobilization of proteins is required to retain their biological activity. As a platform satisfying the requirement, a biotin-modified surface has been widely used because either avidin or biotin-labeled proteins can be selectively immobilized in right orientation through avidin/biotin interaction with high affinity ($K_a \approx 1 \times 10^{15}$ M^{-1}).[3] On the other hand, the serial patterning of proteins is essential to the preparation of protein chips for multiple analysis.[4] To prepare such patterns utilizing biotin/avidin interactions, two kinds of strategies have been developed. First is site-selective immobilization of biotin before protein attachment by using the soft lithography technique[5] or scanning probe microscopy.[6] Although these proved to be versatile protein patterning methods, several drawbacks restrict their application. For example, time consuming in the complex and serial patterning may lower the activity of previously attached proteins under ambient condition. Electrochemical approaches based on this strategy have been also reported.[7] However, their reaction conditions are not compatible with the previously presented protein. They may suffer from ligand contamination bringing about cross adsorption of the protein. The second strategy is a site-selective bioactivation of initially deactivated biotin surface by photo irradiation.[8] In the approach, a whole surface is precoated with deactivated biotin containing a photolabile protecting group, and then only selected sites are activated by the photo irradiation through the photo mask. When the surface is exposed to a solution of a target protein labeled with avidin, the protein is immobilized on the activated sites through avidin linkage. In some cases, however, longer photo irradiation to achieve complete deprotection can cause irreversible damage to the surface, which results in increased nonspecific adsorption of the protein. This should be overcome for the retention of protein activity as well as for higher contrast patterning.

Therefore, it is of great benefit to develop a fast, mild, and protein-friendly method for protein patterning. In this paper, we report a method for protein patterning based on an electrochemically active biotin derivative that generates a bioactive biotin surface by mild electrochemical perturbation. The electrochemical activation proceeds under the buffered aqueous environment at neutral pH. It also allows site-selective generation of bioactive biotin for the immobilization of the target protein by using prepatterned electrode arrays.

Scheme 1 displays the chemical structure of the electrochemically active biotin derivative (**I**) employed in this paper and a proposed

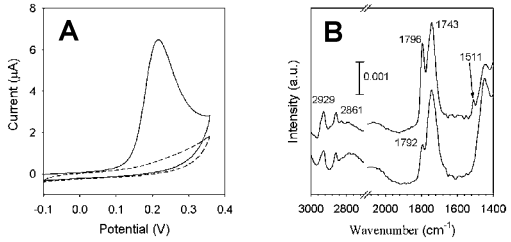
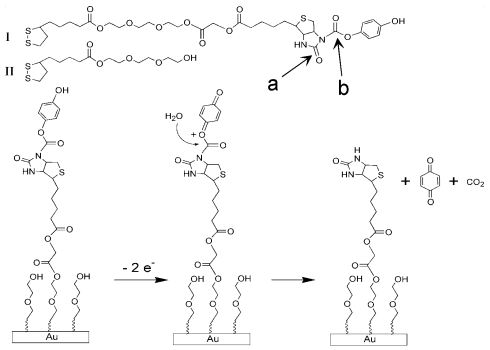


Figure 1. (A) Cyclic voltammogram of **I**-based SAM on a gold electrode in 0.1 M phosphate buffer (pH 7.2) at a scan rate of 50 mV/s. The solid line is the first scan, and the dashed line is the second scan. Reference electrode is Hg/Hg_2SO_4. (B) Grazing angle FT-IR spectra obtained for a freshly prepared **I**-based SAM (top) and after the SAM was subjected to electrochemical reaction (bottom).

Scheme 1. Schematic Representation of the Electrochemically Oxidative Reaction for the Generation of Bioactive Biotin on the HQ-Caged Biotin-Modified Gold Surface



mechanism for the generation of a bioactive biotin surface after electrochemical oxidation. Once the hydroquinone (HQ) moiety is oxidized, it is converted to a benzoquinonium cation. Subsequently, benzoquinone (BQ) and CO_2 are immediately released after nucleophilic acyl substitution by H_2O, and finally, the bioactive biotin surface is generated. To demonstrate the surface reaction proposed in Scheme 1, cyclic voltammetry (CV) was performed with the self-assembled monolayers (SAMs) of compound **I**, prepared by immersion of gold surfaces in 1 mM THF solution of **I** for at least 12 h. Figure 1A shows an irreversible anodic peak at 0.20 V on the first scan and a dramatic disappearance of the peak on the second cycle, representing very fast and nearly complete release of the BQ moiety from the initial surface. For the second cycle, no change in the charging current of the double-layer region, compared to that of the first cycle, indicates that the oxidation potential is mild enough to not destroy the remaining organic SAMs.

Figure 1B shows the grazing angle Fourier transform infrared (FT-IR) spectra of chemical environments on SAM surfaces, which support our view that the electrochemical bioactivation step is very

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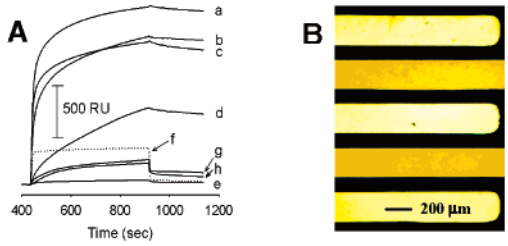

Figure 2. (A) SPR sensorgrams of SA adsorption on various mixed SAMs for Γ_I = (a) 0.25, (b) 0.5, (c) 0.1, (d) 1.0, and (e) 0. Adsorption of SA (f) preblocked with excess free biotin at $\Gamma_I = 0.25$. SPR sensorgrams for SAM surfaces for Γ_I = (g) 0.5 and (h) 1.0 before electrochemical treatments. (B) Optical microscope image of pHRP-SA-patterned IDA.

fast and complete. In the spectrum of **I**-based SAMs, we observed two strong bands at 1743 and 1796 cm^{-1} corresponding to the C═O stretching of three esters and two urethane groups (a and b in Scheme 1), respectively. The peak at 1511 cm^{-1} is characteristic of aromatic C═C stretching of the HQ moiety. The C−H stretching bands of the alkyl chain appear at 2929 and 2861 cm^{-1}. The spectrum of the monolayers after electrochemical reaction in a 0.1 M phosphate buffer solution showed distinct changes from original SAMs.[9] While the peak at 1743 cm^{-1} (C═O stretching in the ester moiety) revealed no significant change, the aromatic C═C stretching at 1511 cm^{-1} disappeared, and the peak intensity at 1796 cm^{-1} (C═O stretching in urethane) was reduced by half of the initial intensity. These results indicate that the HQ moiety was completely detached, and the C═O of "b" was also removed from the **I**-based SAMs. Moreover, the negligible changes in intensity, as well as the position of the C−H stretches, also imply that the other parts of **I** are kept intact. This implication also supports the view that the electrochemical reaction of HQ is mild.

To evaluate biological activity of the activated biotin surface after the electrochemical reaction, we tested a biospecific interaction with streptavidin (SA). Tri(ethylene glycol) ester of thioctic acid (**II**) was used as a diluent of **I**-based SAMs not only to adjust proper density of surface biotin but also to prevent nonspecific adsorption of protein.[10] Under the condition, we optimized the surface density of **I** to maximize SA binding. The density can be easily controlled by changing the mixing ratio of **I** and **II** in adsorbent solutions. The binding of streptavidin on mixed SAMs with various mole fractions of **I** in the solution (Γ_I) was monitored using SPR after the electrochemical oxidation (Figure 2A).[11] When Γ_I was 0.25 and the absolute surface density from CV was 5×10^{-11} mol cm^{-2},[12] the maximum attachment of SA was observed (175 ng cm^{-2}, curve "a"). This density is consistent with that of the previous reports on the maximum binding condition of surface biotin.[8c,13] Curve "e" shows that nonspecific binding of SA was negligible in **II**-based SAMs (2 ng cm^{-2}). Curve "f" supports that these bindings are biospecific. The biospecific interactions between SA and the mixed SAMs before electrochemical bioactivation were negligible (curves "g" and "h"). This SPR study confirms that bioinactive HQ-caged biotin is completely converted into a bioactive biotin surface under mild electrochemical reaction.

We constructed **I**-based SAMs on an interdigitated microelectrode array (IDA) for protein micropatterning. To visualize the pattern, we used poly-(horseradish peroxidase)-conjugated SA (pHRP-SA).[14] Using SPR, we determined Γ_I, presenting maximum pattern contrast between deactivated and activated biotin surfaces, by comparing the amounts of pHRP-SA bound to the surfaces before and after electrochemical activation.[15] Maximum contrast was observed at $\Gamma_I = 0.5$, and thus we employed this condition for micropatterning. Only one electrode of an IDA substrate was electrochemically treated at 0.23 V for 1 min. The resulting substrate was immersed in phosphate-buffered saline solution containing pHRP-SA. After pHRP-SA immobilization, the pattern was visualized by a color change that resulted from catalytic precipitation of 4-chloro-1-naphthol by HRP. Figure 2B shows an optical microscopic image of micropatterned pHRP-SA on IDA. Bright and dark regions correspond to the electrodes before and after electrochemical activation, respectively. The two electrodes highly contrast each other, which indicates that the present approach is very efficient for site-selective protein micropatterning.

In conclusion, we demonstrated that site-specific, as well as site-selective, protein patterning with high contrast could be achieved by means of an electrochemical bioactivation reaction of a rationally designed HQ-caged biotin surface under mild electrochemical conditions. Since the present patterning process, including surface activation and protein attachment, can be conducted under neutral buffer conditions in a short time period, it enables the serial patterning of multiple proteins while retaining the activity of previously attached proteins. Therefore, it might have applications for fabricating protein chips and biomolecular electronic devices.

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Supporting Information Available: Detailed synthetic procedure of compounds **I** and **II**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(11) BIACORE X SPR and SIA Kit Au surfaces were used for SA binding studies. The 1000 RU corresponds to an SA density of 1 ng mm^{-2}.
(12) The absolute surface density of **I** in the mixed monolayers can be easily obtained from integrating the oxidation peak of the HQ of **I** on the CV.
(13) Due to the nonlinear structure of thioctic acid of compound **I**, Γ_I, presenting maximum binding of SA, is higher than the mole fraction of biotin/alkanethiols (∼0.1) of the linear methylene chain in other investigations. Nevertheless, the absolute surface density of biotin is similar to that expected in them. (a) Spinke, J.; Liley, M.; Schmitt, F. J.; Guder, H. J.; Angermaier, L.; Knoll, W. *J. Chem. Phys.* **1993**, *99*, 7012−7019. (b) Nelson, K. E.; Gamble, L.; Jung, L. S.; Boeckl, M. S.; Naeemi, E.; Golledge, S. L.; Sasaki, T.; Castner, D. G.; Campbell, C. T.; Stayton, P. S. *Langmuir* **2001**, *17*, 2807−2816.
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(15) SA binding onto the surfaces before electrochemical activation is also dependent on Γ_I, although the binding constant of them must be much lower than that of HQ-released surface after electrochemical activation.

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