Protein micropatterning based on electrochemically switched immobilization of bioligand on electropolymerized film of a dually electroactive monomer†

Kyuwon Kim,*[a] Jaeyang Hwang,[b] Inwoong Seo,[b] Tae Hwan Youn[c] and Juhyoun Kwak*[b]

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We demonstrate a protein micropatterning method based on electropolymerization of a monomer with two electroactive units, hydroquinone monoester and disulfide, which enables electrochemical ON–OFF switching for immobilization of bioligands on electrodes modified with the electropolymerized film.

The precise and reproducible positioning of biomolecules at designated locations is essential for the fabrication of biologically multifunctional devices such as array-based biochips for diagnosis and electronics.[1,2] Electrochemical patterning methods using preexisted electrode arrays are useful for device fabrication since each electrode can be individually controlled.[3–17] Among these methods, electropolymerization is especially useful for the production of functional surfaces because it entails a rapid one-step procedure, and can be performed on a wide range of electrode materials.[10–17] By contrast, patterning methods based on self-assembled monolayers (SAMs) can only be performed on electrodes amenable to SAM formation.[3–9] In addition, electropolymerization is an attractive technique for biochip fabrication since it allows the discrete patterning of biomolecules at closely spaced microelectrodes.[14–17]

To construct a bioanalytical surface using electropolymerization, a two-step process is generally used.[10] First, a thin polymer film is formed on aimed electrodes *via* electropolymerization of a monomer. Second, bioactive ligands or biomolecules are immobilized on the film *via* a further anchoring reaction. In the latter step, a covalent coupling reaction can be employed, thereby creating strong bonds which prevent the loss of captured molecules. This ability to strongly bind the captured molecules is the reason why a two-step approach is preferred over a one-step process in which target biomolecules are entrapped during the electropolymerization process. However, although the electropolymerization methods developed to date have various characteristics that are advantageous for patterning of biomolecules, they also have several limitations restricting their use. For example, the monomers used have had a single functional group susceptible to electrochemical reactions, which is involved in the electropolymerization. This has meant that the electropolymerized surface does not have an additional function that can be modified electrochemically to trigger the immobilization of a bioligand or biomolecule. By contrast, the electrochemical modification of functional groups on SAMs has been exploited in various studies, and has been shown to be very useful in the micropatterning of biomolecules.[4–9]

In this paper we report for the first time on the use of a dually electroactive monomer for protein micropatterning based on an electropolymerized film. For this purpose, we employed a hydroquinone monoester-conjugated disulfide (HMDS) as the monomer. Fig. 1 shows the chemical structure of the HMDS and the proposed scheme for protein patterning *via* step-by-step modification of an ITO array by chemical and electrochemical treatments. The monomer was synthesized in a similar manner as reported previously.[18] Within the monomer, the hydroquinone

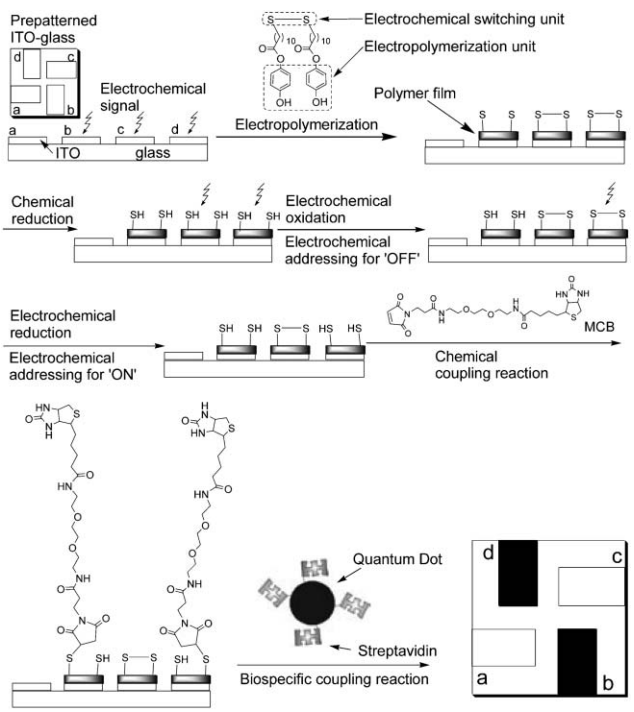

Fig. 1 The chemical structure of HMDS employed in this paper and a proposed scheme for protein patterning by step-by-step modification of the ITO surface by chemical and electrochemical treatments.

[a]*Department of Chemistry, University of Incheon, Incheon 402-749, Korea. E-mail: Kyuwon_Kim@ incheon.ac.kr; Fax: +82 32 770 8238; Tel: +82 32 770 8243*
[b]*Department of Chemistry, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Korea. E-mail: Juhyoun_Kwak@kaist.ac.kr; Fax: +82 42 869 2833; Tel: +82 42 869 2833*
[c]*Interdisciplinary Program in Cognitive Science, Seoul National University, Seoul 151-742, Korea*
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monoester group acts as an electropolymerizable unit and the disulfide group as an electrochemical switching unit. The patterning process therefore involves thin film formation *via* the hydroquinone monoester group, followed by on–off immobilization of biomolecules on the disulfide groups of the resulting polymer film. For this work, a band-type microarray of individually addressable indium–tin–oxide (ITO) electrodes was prepared by a simple microfabrication process (see ESI†).

Fig. 2(a) shows a cyclic voltammogram (CV) for the electropolymerization of HMDS, which shows irreversible anodic peaks near 2.0 V (*vs.* Ag/AgCl as a reference electrode) as continuous scans. As the number of cycles is increased, the anodic current decreases and the peak potential gradually shifts to more positive potential. This can be attributed to the decrease in conductivity of the electrode surface with increasing thickness of the nonconducting polymer film. Our findings are consistent with typical features observed previously for electropolymerized nonconducting polymers.[12–14]

The changes in surface morphology during electropolymerization were investigated using atomic force microscopy (AFM) in the tapping mode (Fig. 2(b)). After one CV cycle, the morphology remains similar to that of bare ITO. As the cycle number increases above one, however, the surface morphology becomes soft. This observation is consistent with the growth of the polymer film as the number of CV cycles increases. The electrically nonconducting nature of the polymer means that its growth is self-limited to be very thin (10–100 nm), in contrast to the virtually unlimited growth of conductive polymers. The thickness measured in our AFM study did not exceed 30 nm, even after seven cycles

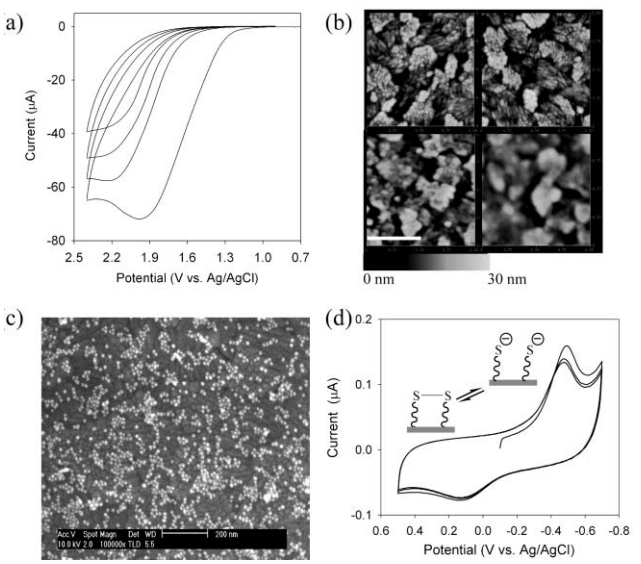

Fig. 2 (a) CV for the electropolymerization of 1 mM HMDS in an acetonitrile solution of 0.1 M TBAP (scan rate = 50 mV s^{-1}). (b) AFM images of the polymer films on ITO electrodes at different potential cyclings (clockwise from top left: 0, 1, 3, 7). Imaging was performed in air at ambient temperature using non-contact mode (scale bar = 500 nm). (c) SEM image of Au nanoparticles on the thin film formed by one cycle sweep of CV. (d) CV for the surface reaction of thiol–disulfide interconversion in an acetonitrile solution of 0.1 M TBAP (scan rate is 50 mV s^{-1}).

The polymer that formed on the surface was investigated using reflectance FTIR spectroscopy. The spectrum exhibited two absorption bands, at 1763 and 1509 cm^{-1}, which are assigned to the C=O stretching vibration of the ester group and the aromatic C=C stretching vibration of the hydroquinone moiety, respectively. The C–H stretching bands of the methylene group of the alkyl chain appear at 2930 and 2853 cm^{-1}. These spectral features are very similar to those reported previously for the SAMs of HMDS analogues.[18] This similarity suggests that the structure of the repeat unit of the polymer formed by electropolymerization is similar to that of the monomer. We found that when the electrode covered in the polymer film was immersed in an aqueous solution of gold nanoparticles, the particles were adsorbed on the polymer surface (Fig. 2(c)). By contrast, the gold nanoparticles did not adsorb on the bare ITO surface under the same conditions.

If the disulfide group is linked to the polymer backbone of the film, the film may be electrochemically active owing to the interconversion reaction between disulfide and thiol.[19] Fig. 2(d) shows the CV for the ITO electrode coated with the film prepared by 3-cycle electropolymerization under potential ranges from 800 to 2400 mV. As expected, the current density was highly dependent on the film preparation conditions such as the cycle number and potential range, which may directly govern the film thickness. The peak intensity did not decrease significantly with increasing cycle number, indicating that the redox reaction is mild and that there is negligible loss of film contents during the reaction. To confirm whether the redox peak is due to the electrochemical reaction of the disulfide moiety, we examined the reduction of the film by tris(2-carboxyethyl)phosphine (TCEP), an agent that efficiently reduces disulfide to thiol.[20] After immersing the film surface in an aqueous solution of 25 mM TCEP for 4 h, the first cathodic scan of the CV from 0 to −700 mV for the film did not exhibit a reduction peak, while a new peak appeared on the second cathodic scan from 500 to −700 mV after the first anodic scan from −700 to 500 mV. These results are the same as those obtained for the film subjected to the electrochemical reaction by holding the film electrode at the reduction potential of −500 mV for 10 s. The observation of similar behavior for these systems confirms that the redox reaction observed in the CV is due to disulfide–thiol interconversion. The current density associated with the electrochemical reaction of the disulfide moiety is much lower than that associated with the electropolymerization step, indicating that the film formed by electropolymerization is very thin. The small thickness of the film is probably due to most of the polymer products having low molecular weights on account of their low degree of polymerization, which causes them to detach from the electrode surface during the growth and cleaning of the film. Collectively, the above results confirm the presence of disulfide on the film surface.

Next we used the new film surface in the patterning of streptavidin (SA) as a model protein on an individually addressable electrode array. To immobilize SA on the thiol-terminated surface, we used maleimide-conjugated biotin (MCB) as a linker because the biospecific interaction between biotin and SA (association constants, $K_a \sim 10^{15}$ M^{-1} in solution) is very strong and the maleimide molecule reacts with thiol to form a stable thioether crosslink.[21,22] By contrast, the maleimide functional group does not react with the disulfide groups. This is an important point for the rational design of our micropatterning

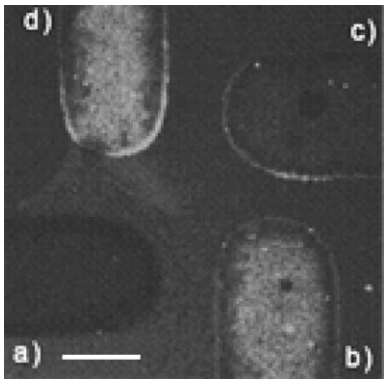

Fig. 3 Fluorescence microscopic image of protein pattern obtained by using the proposed scheme (scale bar = 100 μm).

scheme because electrochemical conversion from disulfide to thiol is intended as the electrochemical 'ON' switch to trigger a further immobilization reaction of targeted molecules, and the reverse reaction corresponds to the 'OFF' switch. If covalent coupling reaction between thiol and maleimide were to occur, the electrochemical activity of the thiol due to the interconversion reaction would gradually disappear. As expected, we found that the anodic peak in the CV for the electrochemically reduced electrode decreased by about 60% as the coupling reaction was completed in phosphate-buffered saline (PBS, pH 7.2) solution containing 10 mM *N*-ethylmaleimide (NEM), which is useful for blocking free thiols. The low yield of the coupling reaction may be attributed to the changes of free thiol to disulfide by natural oxidation and quinone as an oxidizer during the coupling reaction. For the control system, which was identical except it lacked NEM, the peak decrease was negligible.

The procedure for protein patterning on the ITO band microelectrode array was as follows (see Fig. 1). First, thin films were selectively formed on electrodes b, c and d by simultaneous electrochemical addressing of the electrodes so as to promote electropolymerization of HMDS. Next, the whole array was treated with TCEP, which reduces disulfide groups to the thiol form. Among the reduced electrodes, electrodes 'c' and 'd' were subjected to the electrochemical reaction by holding them at the oxidation potential of +300 mV for 20 s, which is the second electrochemical addressing for 'OFF'. After that, electrode 'd' from among the oxidized electrodes was electrochemically reduced by holding it at the reduction potential of −500 mV for 20 s, which is the third electrochemical addressing for 'ON'. Then, the whole array was immersed in the PBS solution containing MCB. The resulting biotin-treated array was exposed to the buffer solution of SA conjugated with a quantum dot as a fluorescence label. Fig. 3 shows a high-contrast fluorescence microscopic image of the protein pattern obtained by using the above scheme. The chemically reduced electrode 'b' and electrochemically reduced electrode 'd', which are expected to have free thiol groups, are both highly fluorescent, whereas the bare electrode 'a' is not. The small amount of fluorescence observed from electrode 'c' may be due to the coupling reaction between MCB and the remaining unoxidized free thiol.

In this study we have demonstrated a protein micropatterning method based on electropolymerization using a monomer with two electroactive units, hydroquinone monoester and disulfide. We showed that the redox reaction of the disulfide group enables ON–OFF switching for the immobilization of target molecules through multiple electrochemical addressing of the film electrode. We believe that our method will open the way for the development of smart surfaces with electrochemical functions that can be switched on demand.

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