Electrochemical cleavage of azo linkage for site-selective immobilization and cell patterning†

Hyun Joo Jung,[a] Hyegeun Min,[b] Hyunung Yu,[b] Tae Geol Lee*[b] and Taek Dong Chung*[a]

Received 29th October 2009, Accepted 1st April 2010
First published as an Advance Article on the web 4th May 2010
DOI: 10.1039/b922611g

A new molecular structure of azobenzene, which can be reduced to the corresponding anilines at a low reduction potential for the site-selective immobilization of biomolecules, is proposed. Redox-active species, enzymes, peptides, and cultured hippocampal neurons can be effectively immobilized on the electrochemically activated surface.

Surface micropatterning of biomolecules such as DNA, peptides and proteins is a key issue in a variety of applications, including multiplexing detection[1] and cellular dynamics.[2] To immobilize biomolecules onto the desired part of a substrate, it is crucial to spatially control surface functionalities. The demand for this technology has stimulated the search for new ways of providing site-selective mechanical, chemical and electrochemical surface activation.[3–5] Among the strategies previously proposed, electrochemical methods are particularly attractive, given the fewer limitations in dimension and the lack of need for mask-aligning, unlike conventional lithographic tools, making it suitable for the patterning of nano-scale electrodes.

There have been many reports of electrochemical strategies for site-selective surface activation. The most typical method is to modify the surface with a redox-active molecular layer such as hydroquinone, which can be transformed to reactive functional moieties through appropriate electrochemical stimulation.[4,5] Mrksich *et al.* modified a gold surface with a hydroquinone monolayer and then oxidized it to benzoquinone, where cyclopentadiene-tagged RGD peptides associated for cell adhesion.[3a] Others involved the anchoring of oxyamine-tagged biomolecules to benzoquinone on the surface.[5] These methods, however, commonly employ a critical step of tagging biomolecules with a reactive group such as cyclopentadienes or oxyamines, which may harm the biological functions of large biomolecules, including proteins.

The electrochemical potential for surface activation is of importance because a positive potential for oxidation among immobilized hydroquinones readily produces oxide layers on the underlying electrodes. This causes a serious problem on a silicon surface, on which many sophisticated functional components can be integrated. Oxide films on a silicon surface make an organic monolayer unstable and electrochemistry unreliable due to the uncontrolled insulating properties involved. Therefore, activation *via* reduction rather than oxidation is preferable to prevent oxidation of the electrode surface. Heath *et al.* introduced a non-oxidative approach by reducing benzoquinone to produce amines on a silicon surface.[6] This method, however, requires a highly negative potential, which may cause undesirable electrochemical side-reactions, and involves complex modification processes.

In the present study, as an alternative to conventional methods, we propose a new molecular structure, azobenzene, which undergoes $4e^-$, $4H^+$ reduction to yield the corresponding anilines at a low reduction potential. Azobenzene is electrochemically reducible *via* the $2e^-$, $2H^+$ process to produce hydrazobenzene.[7] We paid attention to the fact that a further $2e^-$, $2H^+$ process from hydrazobenzene leads to complete cleavage of the hydrazo bond at low pH, resulting in amines which is a useful molecular motif for immobilization of biomolecules.[8] To date, there has been no report of amines being created from an azobenzene-modified gold surface through electrochemical reductive cleavage and used to selectively immobilize biomolecules. The electrochemical cleavage of azobenzene is expected to provide a novel way of selective activation of a specific pattern with a low reduction potential and protecting it from non-specific adsorption by conjugating tri(ethylene glycol) (TEG) at the terminus (Fig. 1).

In Fig. 2, the electrochemical cleavage mechanism of the 3-(4-(phenyldiazonyl)phenoxy)propane-1-thiol (**1**) monolayer, formed on the gold surface through spontaneous chemisorptions,[9] is suggested based on the literature.[7a] The electrochemical behavior of azobenzene was investigated at low pH, in a previously reported potential range.[7a]

As shown in Fig. 3A, the reduction of azobenzene to hydrazobenzene and the oxidation *vice versa* occurs at −0.22 V (*vs.* Ag/AgCl) and 0.08 V in the first scan at pH 2. The anodic peak around 0.08 V indicates the presence of hydrazobenzene, created by the electrochemical reduction of

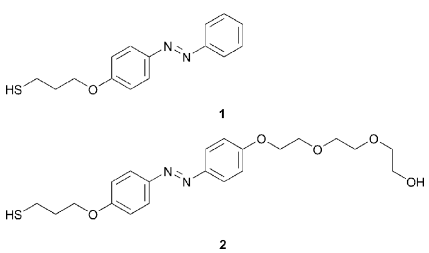

Fig. 1 Molecules employed in this study: 3-(4-(phenyldiazonyl)-phenoxy)propane-1-thiol (**1**), tri-(ethylene glycol)-tethered 3-(4-(phenyl-diazonyl)phenoxy)propane-1-thiol, TEG-tethered azobenzene (**2**).

[a] *Department of Chemistry, Seoul National University, Seoul 151-747, Korea. E-mail: tdchung@snu.ac.kr; Fax: +82-2-887-4354; Tel: +82-2-887-4362*
[b] *Center of Nano-Bio Convergence Research, Division of Convergence Technology, Korea Research Institute of Standards and Science (KRISS), Daejeon 305-600, Korea. E-mail: tglee@kriss.re.kr; Fax: +82-42-868-5252; Tel: +82-42-868-5129*

† Electronic supplementary information (ESI) available: Experimental details. See DOI: 10.1039/b922611g

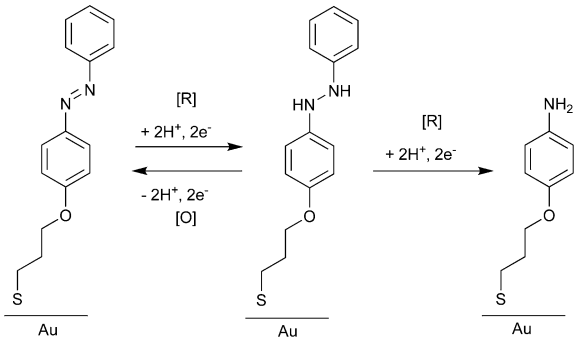

Fig. 2 Electrochemical cleavage mechanism of 3-(4-(phenyldiazonyl)-phenoxy)propane-1-thiol.

azobenzene on the surface. The cyclic voltammogram in the extended potential range (dashed line in Fig. 3B) shows two reduction waves, at −0.22 V and at −0.48 V, during the initial scan in the negative direction. Subsequently, the $2e^-$ and $2H^+$ transfer to the hydrazobenzene is responsible for the latter, where the azo linkages are broken to yield anilines. The absence of the oxidation peak in the reverse scan indicates that azobenzene molecules on the surface are completely cleaved and no hydrazobenzene remained. For the cleavage of azobenzene, we used a potentiostatic pulse rather than potential cycling, to prevent undesirable reactions of the produced aniline monolayer which can be re-oxidizable.[10] After a potentiostatic pulse was optimally applied at −0.4 V for 15 s, the absence of the anodic peak due to the oxidation of hydrazobenzene was verified by cyclic voltammetry.

Fig. 3C shows the electrochemical behavior of TEG-tethered azobenzene (**2**). TEG had covalently bonded to

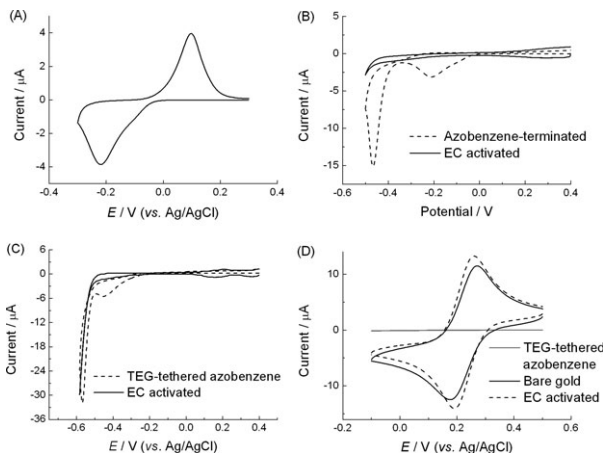

Fig. 3 Cyclic voltammograms of an azobenzene-modified gold electrode in Britton–Robinson buffer at pH 2 at a scan rate of 50 mV s^{-1}. (A) Cyclic voltammogram of azobenzene. Reduction of azobenzene to hydrazobenzene at −0.22 V and oxidation *vice versa* at 0.08 V occur. (B) Cyclic voltammogram in a wider potential range in which two peaks appear at −0.22 V and −0.48 V (dashed). Cyclic voltammogram after potentiostatic stimulation (solid). (C) Cyclic voltammogram of TEG-tethered azobenzene, in the same conditions (dashed). Cyclic voltammogram after potentiostatic stimulation (solid). (D) Cyclic voltammograms of 1 mM potassium ferricyanide in 0.1 M potassium chloride aqueous solution on bare gold (solid black). TEG-tethered azobenzene (solid grey) and electrochemically activated electrodes by applying potentiostatic pulse for 15 s (dashed).

azobenzene and was intended to prevent organic monolayers from non-specific adsorption. The first reduction occurs at −0.44 V, which is a larger negative potential than on the azobenzene-terminated surface. This is not surprising, because an extra potential difference is required, due to the additional layer of the terminal TEG, which possibly provides a less proton-sufficient environment around the azo groups than in the bulk solution. Nonetheless, the entire process of reductive cleavage requires no significant modification in the optimal condition for potentiostatic activation, except a little longer duration. A complete reduction of azobenzenes was also confirmed by the absence of the oxidation peak after the potentiostatic pulse in the cyclic voltammogram. The cyclic voltammograms of ferricyanide ions before and after electrochemical activation (Fig. 3D) show no redox wave from the TEG-tethered azobenzene electrode, which has long chains blocking the electron transfer. After the electrochemical treatment, however, highly reversible electrochemical behavior is observed on the activated surface. The reversibility of the cyclic voltammogram from the activated surface appears better than that from the bare gold electrode. This is because the electron transfer was facilitated by an electrostatic interaction between the negatively charged ferricyanide ions and the positively charged amines on the activated surface.[11] The presence of amines was confirmed by conjugating electroactive ferrocene on the activated surface *via* a Schiff base formation using ferrocenecarboxaldehyde (FCA).[12] A detailed quantitative analysis of surface-bound ferrocene is provided (see ESI†). For further characterization, we analyzed the surface by time of flight secondary ion mass spectrometry (Tof-SIMS), polarized infrared external reflectance spectroscopy (PIERS), and a surface wettability test (see ESI†). The conjugation ability with proteins was demonstrated by horse radish peroxidases (HRPs) immobilized exclusively on the electrochemically treated surface using a homobifunctional NHS crosslinker, BS^3 (Fig. S1 in ESI†).

Various cells can also be cultured on the site-specifically activated area, using the proposed method. For applications such as electrophysiological recording and stimulation, it is crucial to control neuronal cell attachment and growth on conductive materials. We therefore cultured primary hippocampal neurons on the electrochemically activated surface from the TEG-terminated azobenzene (**2**) layer, which prevents the non-specific adhesion of cells. Cys-Gly-Gly (CGG)-linked Ile-Lys-Val-Ala-Val (IKVAV) peptide was conjugated to the electrochemically activated surface using a heterobifunctional NHS-maleimide linker, Sulfo-GMBS. IKVAV is a hippocampal neuron adhesive peptide that originates from laminin, a well-known cell-adhesive extracellular matrix.[13] CGG linker was used to reserve the cell-adhesion function of IKVAV by keeping amine groups of *N*-terminus and lysine intact. As shown in Fig. 4A, hippocampal neurons with growing neurites were rarely found on the TEG-terminated azobenzene monolayer. On the other hand, healthy neurons with extensive neurites were grown on the IKVAV-conjugated, electrochemically activated surface (Fig. 4B). In this experiment, a single piece of gold-deposited wafer was partially immersed into the solution, where electrochemical activation would take place. Only the region dipped in the solution was allowed for

the growth of hippocampal neurons. Fig. 4C clearly shows the contrast between electrochemically stimulated and non-stimulated regions, which are found in the lower and upper parts of the boundary, respectively. Although the entire surface of both the activated and non-activated areas had been exposed to linker and peptide solutions, hippocampal neurons grew only in the activated area. Importantly, the selective spatial control of neuronal growth on the desired surface pattern has a special meaning in terms of recent studies on artificial neural networks, based on surface-patterned neurons[14] and the nano-interface between neurons and electrodes.[15] The proposed method allows for the culturing and patterning of neurons on a desired surface with an electrochemically addressable dimension, possibly as small as the sub-micron level.

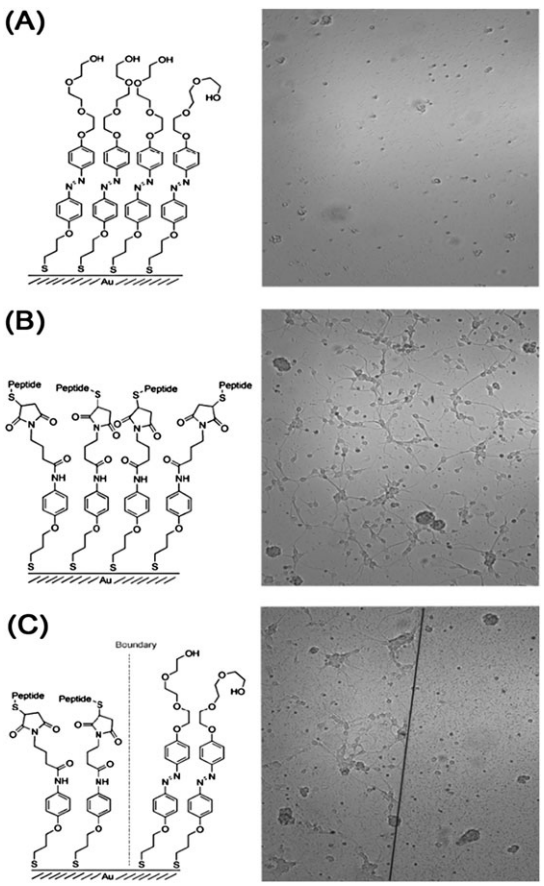

Fig. 4 Hippocampal neuronal cells two days after seeding on the TEG-tethered azobenzene surface (A), on the electrochemically activated and then CGG-IKVAV conjugated surface (B). (C) Comparison in one chip between just the **2**-coated surface and electrochemically stimulated region. By immersing part of the **2**-coated gold surface into the electrolytic solution, only the region dipped in the solution was electrochemically activated; it was there that the neuronal cells later adhered and grew. The black solid line is the meniscus of the solution, which serves as a boundary between the electrochemically activated and non-activated regions.

In summary, we suggest herein a new molecular scheme for site-selective protein immobilization and cell patterning on the basis of electrochemical azo-cleavage. The proposed system offers valuable advantages including a reductive cleavage mechanism that is free from the oxidation of electrode surfaces, and the generation of an amine surface favorable for immobilization of biomolecules. It should be noted that the reductive cleavage strategy is useful with silicon surfaces, where patterning strategies should be applied for more sophisticated applications.

This work was supported in part by the Korea Science and Engineering Foundation (KOSEF) grants funded by the Korea government (MEST) (No. R11-2007-012-02002-0(2009)), by the grant from the Industrial Source Technology Development Program (10033657) of the Ministry of Knowledge Economy (MKE) of Korea, and the Nano/Bio Science & Technology Program (M10536090001-05N3609-00110) of the Ministry of Education, Science and Technology (MEST), South Korea.

Notes and references

1 (*a*) P. Jonkheijm, D. Weinrich, H. Schroder, C. M. Niemeyer and H. Waldmann, *Angew. Chem., Int. Ed.*, 2008, **47**, 9618; (*b*) G. MacBeath and S. L. Schreiber, *Science*, 2000, **289**, 1760; (*c*) G. M. Whitesides, E. Ostuni, S. Takayama, X. Jiang and D. E. Ingber, *Annu. Rev. Biomed. Eng.*, 2001, **3**, 335.
2 (*a*) M. Mrksich, C. S. Chen, Y. Xia, L. E. Dike, D. E. Ingber and G. M. Whitesides, *Proc. Natl. Acad. Sci. U. S. A.*, 1996, **93**, 10775; (*b*) D. K. Hoover, E. W. L. Chan and M. N. Yousaf, *J. Am. Chem. Soc.*, 2008, **130**, 3280.
3 (*a*) M. N. Yousaf, B. T. Houseman and M. Mrksich, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 5992; (*b*) Y. Kwon, Z. Z. Han, E. Karatan, M. Mrksich and B. K. Kay, *Anal. Chem.*, 2004, **76**, 5713.
4 K. Kim, M. Jang, H. Yang, E. Kim, Y. T. Kim and J. Kwak, *Langmuir*, 2004, **20**, 3821.
5 E. W. L. Chan, S. Park and M. N. Yousaf, *Angew. Chem., Int. Ed.*, 2008, **47**, 6267.
6 R. D. Rohde, H. D. Agnew, W.-S. Yeo, R. C. Bailey and J. R. Heath, *J. Am. Chem. Soc.*, 2006, **128**, 9518.
7 (*a*) H.-Z. Yu, Y.-Q. Wang, J.-Z. Cheng, J.-W. Zhao, S.-M. Cai, H. Inokuchi, A. Fujishima and Z.-F. Liu, *Langmuir*, 1996, **12**, 2843; (*b*) W.-W. Zhang, X.-M. Ren, H.-F. Li, C.-S. Lu, C.-J. Hu, H.-Z. Zhu and Q.-J. Meng, *J. Colloid Interface Sci.*, 2002, **255**, 150.
8 (*a*) L. Holleck, S. Vavricka and M. Heyrovsk, *Electrochim. Acta*, 1970, **15**, 645; (*b*) Z. Mandic, B. Nigovic and B. Simunic, *Electrochim. Acta*, 2004, **49**, 607; (*c*) A. Cyr, P. Huot, G. A. Belot and J. Lessard, *Electrochim. Acta*, 1990, **35**, 147.
9 J. C. Love, L. A. Estroff, J. K. Kriebel, R. G. Nuzzo and G. M. Whitesides, *Chem. Rev.*, 2005, **105**, 1103.
10 (*a*) J. Lukkari, K. Kleemola, M. Meretoja, T. Ollonqvist and J. Kankare, *Langmuir*, 1998, **14**, 1705; (*b*) R. C. Schomburg and R. L. McCarley, *Langmuir*, 2001, **17**, 1983.
11 (*a*) K. Takehara, H. Takemura and Y. Ide, *Electrochim. Acta*, 1994, **39**, 817; (*b*) C. Miller, P. Cuendet and M. Graetzel, *J. Phys. Chem.*, 1991, **95**, 877.
12 L. Y. S. Lee, T. C. Sutherland, S. Rucareanu and R. B. Lennox, *Langmuir*, 2006, **22**, 4438.
13 S. Lu, A. Bansal, W. Soussou, T. W. Berger and A. Madhukar, *Nano Lett.*, 2006, **6**, 1977.
14 A. Krogh, *Nat. Biotechnol.*, 2008, **26**, 195.
15 (*a*) Z. Yu, T. E. McKnight, M. N. Ericson, A. V. Melechko, M. L. Simpson and B. Morrison, *Nano Lett.*, 2007, **7**, 2188; (*b*) F. Patolsky, B. P. Timko, G. Yu, Y. Fang, A. B. Greytak, G. Zheng and C. M. Lieber, *Science*, 2006, **313**, 1100.