Electronic Supplementary Information

Electrochemical Cleavage of Azo Linkage for Site-selective Immobilization of Biomolecules and Cell Patterning

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Experimental

Materials. 3-(4-(phenyldiazonyl)phenoxy) propane-1-thiol was synthesized according to the literature[16] by CosBiotech (Daejeon, South Korea) and used as received. TEG-tethered azobenzene, ((E)-2-(2-(4-((4-(3-mercaptopropoxy)phenyl)diazenyl)phenoxy)ethoxy)ethanol), was synthesized and provided by Medigen (Daejeon, South Korea). The synthesis process is described, in detail, in the Supporting Information. CGG-IKVAV peptide was synthesized by Anygen (Gwangju, South Korea). Absolute ethanol (Merck, USA), boric acid (Aldrich, USA), acetic acid (Aldrich), hydrogen peroxide (J.T. Baker, USA), phosphoric acid (Daejung, South Korea), potassium chloride (Daejung), potassium ferricyanide (Daejung), sulfuric acid (Daejung), nitric acid (Daejung), sodium hydrogen phosphate (Daejung), sodium dihydrogen phosphate (Daejung), sodium chloride (Daejung), sodium hydroxide (Daejung), ferrocenecarboxaldehyde (Aldrich), 4-chloro-1-naphtol (Aldrich), horse radish peroxidase (Aldrich) were used as received. A homogeneous bifunctional linker, bis[sulfosuccinimidyl] suberate (BS^3) and a heterogeneous bifunctional linker, *N*-[γ-maleimidobutyryloxy]sulfosuccinimide ester (Sulfo-GMBS) were purchased from Pierce (USA). For

primary cell culture, papain (Worthington, USA), Hank's balanced salt solution (Invitrogen, USA), penicillin/streptomycin (Invitrogen), sodium pyruvate (Aldrich), HEPES (Calbiochem, USA), DNase I (Aldrich), fetal bovine serum (Invitrogen), L-glutamic acid (Aldrich), neurobasal A (Invitrogen), B-27 supplement (Invitrogen), glutamax (Invitrogen) were used as received. All media and buffers were mixed and used as previously reported.[S1] 3-(4-(phenyldiazonyl)phenoxy) propane-1-thiol was synthesized according to the literature by CosBiotech (Daejeon, South Korea) and used as received.[S11] TEG-tethered azobenzene, ((E)-2-(2-(4-((4-(3-mercaptopropoxy)phenyl)diazenyl)pheno--xy)ethoxy)ethanol), was synthesized and provided by Medigen (Daejeon, South Korea). The synthesis process is described in detail (Scheme S1). The CGG-IKVAV peptide was synthesized by Anygen (Gwangju, South Korea).

Electrochemical measurements. All experiments were performed using CHI-660A and CHI-601B potentiostats (CH Instrument, TX, USA). Gold was sputtered 100 nm thick onto a wafer with an adhesive layer of 3 nm thick chromium; it was purchased from Kmac (Daejeon, South Korea) and used as a working electrode. A platinum wire and Ag/AgCl served as counter and reference electrodes, respectively. Self-assembled monolayers on a gold electrode were formed by immersing all substrates into a 1 mM thiol solution in ethanol for 40 h. A home-made electrochemical cell was used to conduct electrochemical experiments where a platinum wire counter electrode and an Ag/AgCl reference electrode. The geometric area of the gold electrode exposed to the solution was 0.066 cm^2. The supporting electrolyte was Britton Robinson buffer deoxygenated by nitrogen purging for 20 min. Britton Robinson buffer is a mixed solution of 0.04 M boric acid, 0.04 M acetic acid and 0.04 M phosphoric acid, and pH was adjusted to desired value by adding 0.2 M sodium hydroxide.

Time of Flight Secondary Ion Mass Spectrometry (ToF-SIMS). ToF-SIMS spectra were obtained by using a ToF-SIMS V instrument (ION-TOF GmbH, Germany) with 25-keV Bi^+ primary ions. The primary ion source was operated with an average current of 0.72 pA, a pulse width of 19.5 ns, and a repetition rate of 6.7 kHz. Positive ion spectra were acquired on three points with an area of 200 × 200 μm^2 on each sample, while maintaining the primary ion dose of 5×10^{11} ions cm^{-2}. The positive

ion spectra were calibrated using the H^+, H_2^+, CH_3^+, $C_2H_3^+$, and $C_3H_5^+$ peaks. The spectra were normalized by the corrected total ion intensities, and the intensities of each molecular ion peak were compared. The mass resolution M / ΔM was more than 8000 between m/z 800 and m/z 1000.

Polarized infrared external reflectance spectroscopy (PIERS). PIERS spectra were acquired in a single reflection mode with a Thermo Nicolet fourier transform infrared spectrometer (Nexus 6700 FT-IR). This instrument was equipped with an advanced grazing angle (AGA) apparatus for grazing-angle specular reflectance IR spectroscopy and a mercury-cadmium-telluride (MCT) detector cooled with liquid nitrogen. The p-polarized light was incident at 80° relative to the surface normal of the gold substrate. All spectra were averaged after 128 scans at a resolution of 2 cm^{-1} with a stream of >99.99% nitrogen gas to purge the chamber.

Contact Angle Measurement. The water contact angles were measured from sessile drops by lowering a 1 μL drop from a syringe needle onto the functionalized gold surface (CAM100, Chang-Kyung Enterprise Corp., Seoul, South Korea). All measurements were repeated five times and averaged.

Immobilization of Ferrocenecarboxaldehyde (FCA) and Horse Radish Peroxidase (HRP). For immobilization of ferrocene, the electrochemically activated substrate was immersed in 0.19 g of FCA in 40 mL ethanol for 40 min. For immobilization of HRP, bis[sulfosuccinimidyl] suberate (BS^3) was used as a linker. A 1 mM linker solution in 20 mM PBS (pH 7.4) was prepared and reacted with the substrates for 30 min. The substrates were taken out from the linker solution, washed with PBS and immersed in a 1 mg mL^{-1} of HRP solution in 20 mM PBS for 30 min. Then they were washed with PBS and DI water thoroughly.

Hippocampal Neuronal Cell Culture. The CGG-IKVAV peptide was immobilized on the electrochemically activated surface using a linker, *N*-[γ-maleimidobutyryloxy]sulfosuccinimide ester (Sulfo-GMBS). After electrochemical activation, all substrates were immersed in a 2 mM linker solution in PBS for 30 min, and then washed with PBS and water thoroughly. Then the substrates were immersed in a 1 mM peptide solution for 1 h, and then washed with PBS and DI water, and immersed in 70% ethanol overnight for sterilization. The next day, the substrates were washed with

sterilized water three times and totally dried in clean bench. The dissociated hippocampal neurons were prepared from embryonic rat (E18) with an approximate density of 2×10^5 cells mL^{-1} as described previously.[1] They were plated at the concentration of 750 cells mm^{-2} in Neurobasal medium supplemented with 2% B-27 (*v*/*v*), 0.25% Glutamax (*v*/*v*), 1% penicillin-streptomycin (*v*/*v*) and 25 μM L-glutamate, then grown at 37℃ in a humidified 5% CO_2 atmosphere. After 3 h, medium was changed with Neurobasal without L-glutamate.

Synthesis of (E)-2-(2-(4-((4-(3-mercaptopropoxy)phenyl)diazenyl)phenoxy)ethoxy)ethanol.

2-(2-(2-Chloroethoxy)ethoxy)ethyl benzoate (1): To a solution of 2-(2-(2-chloroethoxy)ethoxy)ethanol (3.0 g, 17.80 mmol) in CH_2Cl_2 (100 mL) was added pyridine (2.8 g, 35.60 mmol) and benzoly chloride (3.0 g, 21.36 mmol) successively. After stirring for 6 h at RT, reaction mixture was washed with water, brine and 1 M HCl. The separated organic layer was dried over $MgSO_4$ and evaporated to give a residue. Flash column chromatography (Hex : EA = 5 : 1, Rf = 0.7 in Hex : EA = 1 : 1) gave the product as an oil (4.73 g, 17.34 mmol, yield = 98%). See scheme S1.

S-3-Bromopropyl ethanethioate (2): A mixture of 1,3-dibromopropane (5.0 g, 24.76 mmol) and potassium thioacetate (2.6 g, 22.29 mmol, 0.9 eq) in THF (100 mL) was refluxed for 1 h and stirred overnight at RT. The solvent was removed in *vacuo* and the residue was diluted with CH_2Cl_2, and filtered through a Celite pad to remove inorganic salts. The filtrate was evaporated to give a residue. Silica gel column chromatography (Hex : EA = 9 : 1, Rf = 0.7 in Hex : EA = 5 : 1) gave the product as an oil (2.65 g, 13.44 mmol, yield = 54%). See scheme S1.

4,4-(Diazene-1,2-diyl)diphenol (3): To a solution of 4-aminophenol (10.7 g, 98.18 mmol) dissolved in 1 N HCl solution (170 mL), a solution of potassium nitrite (11.3 g, 132.43 mmol, dissolved in 34 mL H_2O) solution at 0 ℃ was added dropwise with constant stirring. The mixture was diluted by adding pre-cooled methanol (340 mL). In a separate flask, phenol (9.2 g, 98.18 mmol) and potassium hydroxide (10.4 g, 185.71 mmol, 1.9 eq) were dissolved in methanol (48 mL) and cooled to 0℃. This phenolate solution was added dropwise under constant stirring to the flask containing diazonium salt. The resulting red solution was stirred for additional 24 h. 1 N HCl solution was added to precipitate the crude product, which was collected by filtration. The crude product was then

recrystallized from glacial acetic acid to give the product as a red-black solid (9.1 g, 42.48 mmol, yield = 43%). See scheme S1.

2-(2-(2-4-((4-Hydroxyphenyl)diazenyl)phenoxy)ethoxy)ethoxy)ethyl benzoate (4) : To a solution of compound **3** (5.0 g, 23.36 mmol), potassium carbonate (11.8 g, 85.19 mmol) and potassium iodide (0.2 g, 1.28 mmol) in DMF (50 mL) was added dropwise with a solution of the compound **1** (3.5 g, 12.83 mmol) in DMF (15 mL). After stirring overnight at 80℃, solvent was removed in *vacuo* and the residue was dissolved in CH_2Cl_2 (50 mL) and the solution was washed with water three times. The organic layer was dried over $MgSO_4$ and evaporated to give a residue. Silica gel column chromatography (Hex : EA = 3 : 1 to 2 : 1, Rf = 0.1 in Hex : EA = 2 : 1) gave the product as a yellow solid (1.9 g, 4.17 mmol, yield = 32.5%). See scheme S1.

2-(2-(2-4-((4-(3-(Acetylthio)propoxy)phenyl)diazenyl)phenoxy)ethoxy)ethoxy)ethyl benzoate (5) : To a solution of compound **4** (750 mg, 1.66 mmol) and S-3-bromopropyl ethanethioate (**2**) (656 mg, 3.33 mmol) in DMF (15 mL) were added potassium carbonate (918 mg, 6.64 mmol) and potassium iodide (28 mg, 0.16 mmol). After stirring overnight at 80℃, solvent was removed in *vacuo* and the residue was dissolved in CH_2Cl_2 and the solution was washed with water three times. The organic layer was dried over $MgSO_4$ and evaporated to give a residue. Silica gel column chromatography (Hex : EA = 3 : 1 to 2 : 1, Rf = 0.6 in Hex : EA = 1 : 1) gave the product as a yellow solid (800 mg, 1.41 mmol, yield = 85%). See scheme S1.

2-(2-(2-4-((4-(3-Mercaptopropoxy)phenyl)diazenyl)phenoxy)ethoxy)ethoxy)ethanol (6): The compound **5** (750 mg, 1.66 mmol) was added to a solution of NaOH (750 mg, 1.66 mmol) in MeOH (5 mL) and the resulting mixture was stirred at RT for 4 h. After evaporation of MeOH in *vacuo*, the residue was added with water (50 ml), and extracted with CH_2Cl_2 (2 x 50 mL). The combined organic layers were dried over $MgSO_4$ and evaporated to give a residue. Silica gel column chromatography (MC : EA = 4 : 1, Rf = 0.3 in Hex : EA = 1 : 2) gave an impure product which was further purified by recrystallization from MeOH to give the product as a yellow solid (196 mg, 0.47 mmol, yield = 33%). See Scheme S1.

Quantification of the Immobilized Ferrocene.

Figure S1(a) shows the cyclic voltammograms of ferrocene immobilized on the activated surface at scan rates of 10–100 mV s^{-1}. The averaged value of peak potentials is consistent with the reported value of 0.36 V on the closely packed ferrocenylalkylthiolate-terminated gold surface. Additionally, both anodic and cathodic peak currents were linearly dependent on scan rates, indicating that the redox species, ferrocene, had bound to the surface. The surface density of redox-active ferrocenyl moieties was estimated by integrating the anodic wave of cyclic voltammograms. Taking into account of the facts that the number of electrons involved in electron transfer is one and the geometric area of the electrode is 0.066 cm^2, the calculated surface density of ferrocene was 1.5×10^{-11} mol cm^{-2}. The theoretical maximum value is 4.5×10^{-10} mol cm^{-2}, assuming that the effective ferrocene diameter is 0.66 nm.[S2] The calculated coverage of ferrocene is about 3.3%, which is comparable to the reported value of the conjugated ferrocene on the electrochemically activated surface.[S3] Relatively low coverage may be resulted from the low conjugation yield as well as side reactions in the reduction process, including hydrolysis, benzidine rearrangements and so on.[S4-S6]

Time of Flight Secondary Ion Mass Spectrometry (ToF-SIMS).

The thiol self-assembled monolayers (SAMs) chemisorbed on gold substrates can be identified with the molecular or gold-molecular cluster secondary ions, $Au_x(M \pm zH)_y^{\pm}$, obtained from ToF-SIMS spectra, where M is the complete thiol molecule (*e.g.* $M_{azo} = C_{15}H_{16}SN_2O$, $M_{aniline} = C_9H_{13}SNO$), x, z = 0, 1, 2, …, and y = 1, 2, ….[S7-S10] Figure S2 shows positive ToF-SIMS spectra of azobenzene-modified gold substrates before and after the electrochemical activation. Initially, a dominant $Au(M_{azo})^+$ peak at *m/z* 469 exist on an azobenzene-modified surface. The small peak at *m/z* 380 in Figure S2a, was regarded as the background rather than the peak of aniline molecular cluster ion, $Au(M_{aniline})^+$. During the electrochemical activation, azobenzene was reduced to aniline through the cleavage of azo linkage. After electrochemical stimulation for 4 s (Figure S2b) and 7.5 s (Figure S2c), the $Au(M_{azo})^+$ peak kept present, but completely disappeared after azobenzene was fully reduced to aniline in 15 s (Figure S2d).

To quantitatively monitor the reduction process of azobenzene to aniline, the intensities of $Au(M_{azo})^+$ and $Au(M_{aniline})^+$ peaks were plotted *versus* the duration of electrochemical stimulation as shown in Figure S3. The intensity of $Au(M_{aniline})^+$ peak gradually increased as electrochemical stimulus was applied for longer duration, then eventually reached to its maximum in 7.5 s. However, the standard deviation of the intensities of $Au(M_{aniline})^+$ peak, which was obtained from three points on the surface, was lowered below 10 %, after the reduction completes in 15 s.

Polarized Infrared External Reflectance Spectroscopy (PIERS).

For the surface analysis by IR spectroscopy, only transition dipoles with a component perpendicular to the surface normal can be observed, according to the selection rule of grazing angle reflectance. Figure S4 presents how IR spectra of the azobenzene-modified gold surface changes with electrochemical reduction in the 3350 ~ 3200 and 1680 ~ 1200 cm^{-1} region. Careful spectral analysis in the low frequency region around 1500 and 1250 cm^{-1} was performed by fitting high resolution spectra with Gaussian modes. As shown in Figure S4a, before electrochemical stimulation, peaks associated with φ-H (~1600 cm^{-1} / 1502 cm^{-1}) and φ-N stretching (1243 cm^{-1}) were observed on an azobenzene-modified surface. After electrochemical activation for 4 s (b), 7.5 s (c), and 15 s (d), however, several new peaks at 3280 cm^{-1} (NH_2 stretching) and 1512 cm^{-1} (in-plane bending of NH_2) appeared and those at 1600, 1502 cm^{-1} (φ-H stretching) and 1243 cm^{-1} (φ-N stretching) bands decreased. As the absorption intensity of φ-N mode decreased, the peak at 1258 cm^{-1} (φ-O mode) became apparent. The result suggested that amines was produced *via* the cleavage of azobenzenes. Figure S5 compares the integrated absorption intensities of major peaks, as a function of the duration of electrochemical activation. Most peaks except 1258 cm^{-1} (φ-O mode) showed a significant increase (NH_2) or decrease (φ-H and φ-N), and then gradually reached to the steady state as the electrochemical reduction completed in 15 s. The variation of absorbance meant that the molecular composition was rapidly changed at the initial stage of electrochemical stimulation (4 s), being further transformed at the middle (7.5 s) and finally stabilized (15 s).

Contact Angle Measurements.

An azobenzene-modified surface is so hydrophobic because of the terminal benzene rings, while an aniline-modified surface is mostly positively charged and expected to be hydrophilic due to the high p*K*a value of anilines. With the cleavage of surface-bound azobenzenes, the surface is likely to be wet, as its hydrophobic property is changed to be hydrophilic. Therefore, the water contact angles, which were measured on an azobenzene-modified, electrochemically activated for 15 s at -0.4 V (vs. Ag/AgCl) and aniline-modified surfaces, were compared. As shown in Table S2, The measured contact angle of the electrochemically activated gold surface was not only similar to that of the aniline-modified surface, also smaller than that of the azobenzene-modified and bare gold surface, indicating that the surface wetting property was changed to being hydrophilic by electrochemical stimulation. The dynamic change of surface functionalities from azobenzenes to anilines enables us to control surface wettability on demands.

References

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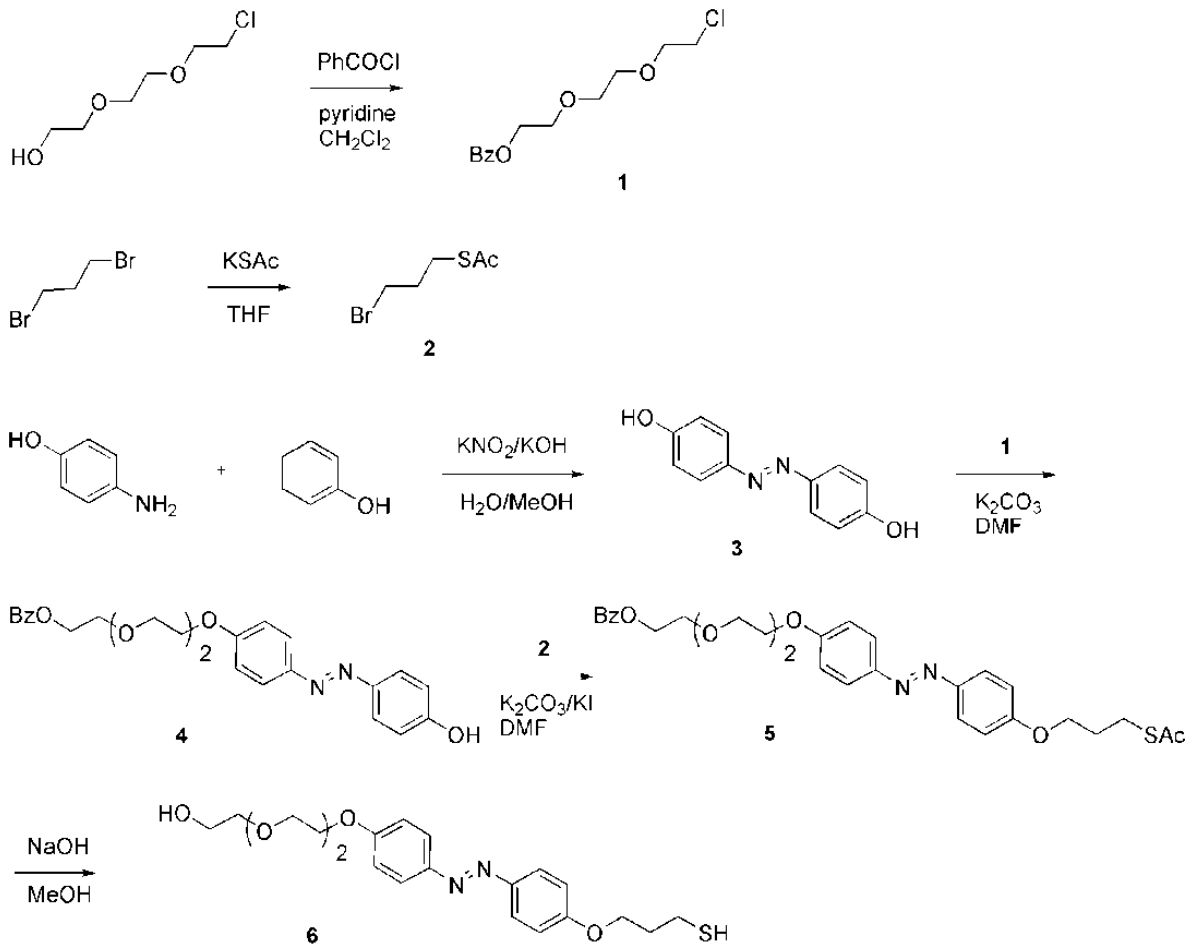

Scheme S1. Synthesis of (E)-2-(2-(4-((4-(3-mercaptopropoxy)phenyl)diazenyl)phenoxy)ethoxy)et--hanol

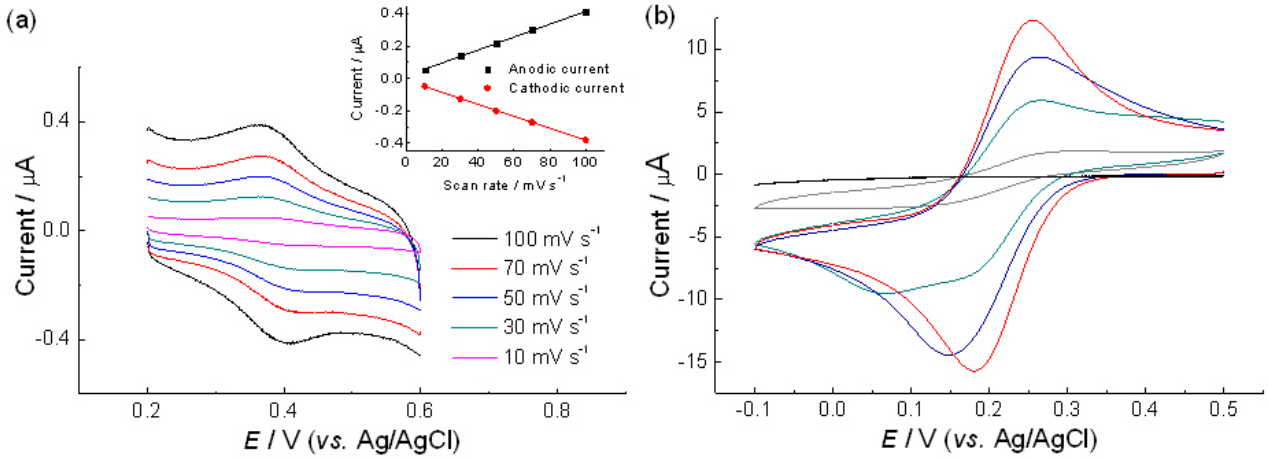

Figure S1. (a) Cyclic voltammograms of ferrocene immobilized on an electrochemically activated electrode, at a variety of scan rates. Linear dependence of cathodic and anodic peak currents on scan rate (inset) (b) Cyclic voltammograms of 1 mM ferricyanide obtained at a respective conjugation step for HRP immobilization. Cyclic voltammograms recorded after deposition of the monolayer on the electrode (black), electrochemical activation (red), immobilization of homobiofunctional linker, BS^3 (blue), conjugation of HRPs (green), and precipitation of 4CN by HRPs (grey). The activated circular region with HRPs was visualized by a colour change resulting from the precipitation of 4-chloro-1-naphtol (4CN) produced by HRP catalysis.

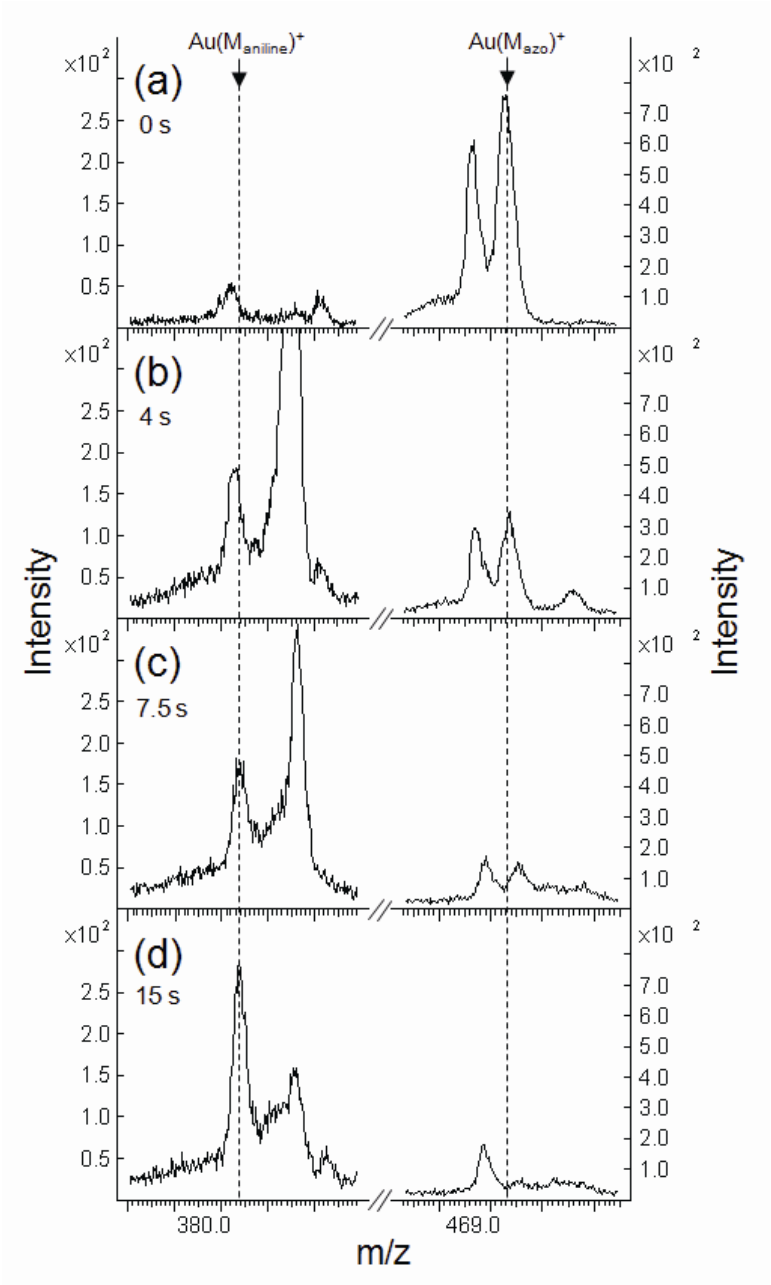

Figure S2. Positive ion ToF-SIMS spectra (a) before electrochemical activation, after electrochemical activation for (b) 4 s, (c) 7.5 s, and (d) 15 s. The spectra of aniline- and azobenzene-modified surface show the characteristic molecular cluster peaks, $Au(M_{azo})^+$ at *m/z* 380 and $Au(M_{aniline})^+$ at *m/z* 469, respectively.

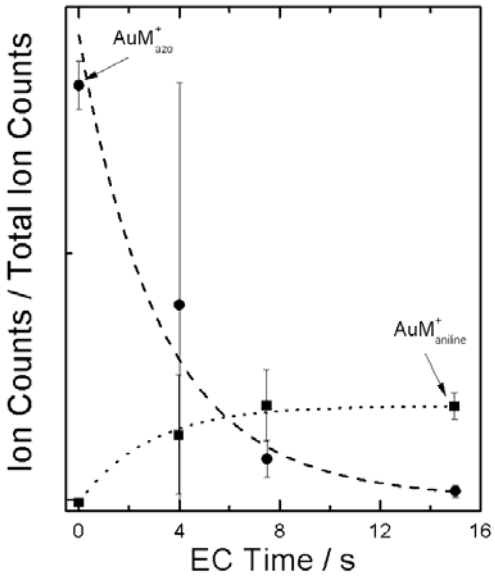

Figure S3. The plots of intensities of $Au(M_{azo})^+$ (circles) with a fitted line (dashed line) and $Au(M_{aniline})^+$ (squares) with a fitted line (dotted line), which were normalized by total ion counts, *versus* the duration of electrochemical stimulation.

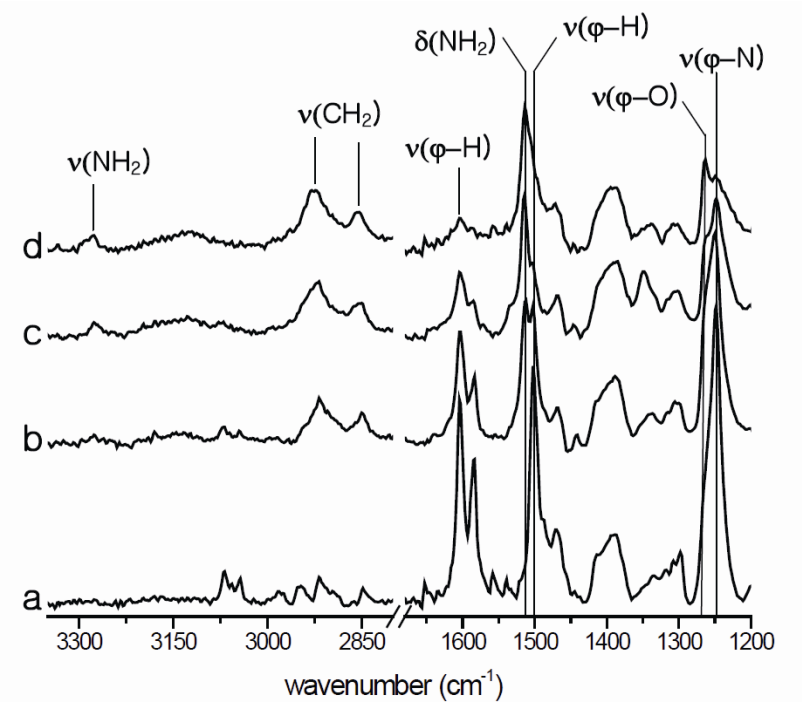

Figure S4. FT-IR spectra on (a) the azobenzene-modified and electrochemically activated gold surface for (b) 4 s, (c) 7.5 s, and (d) 15 s.

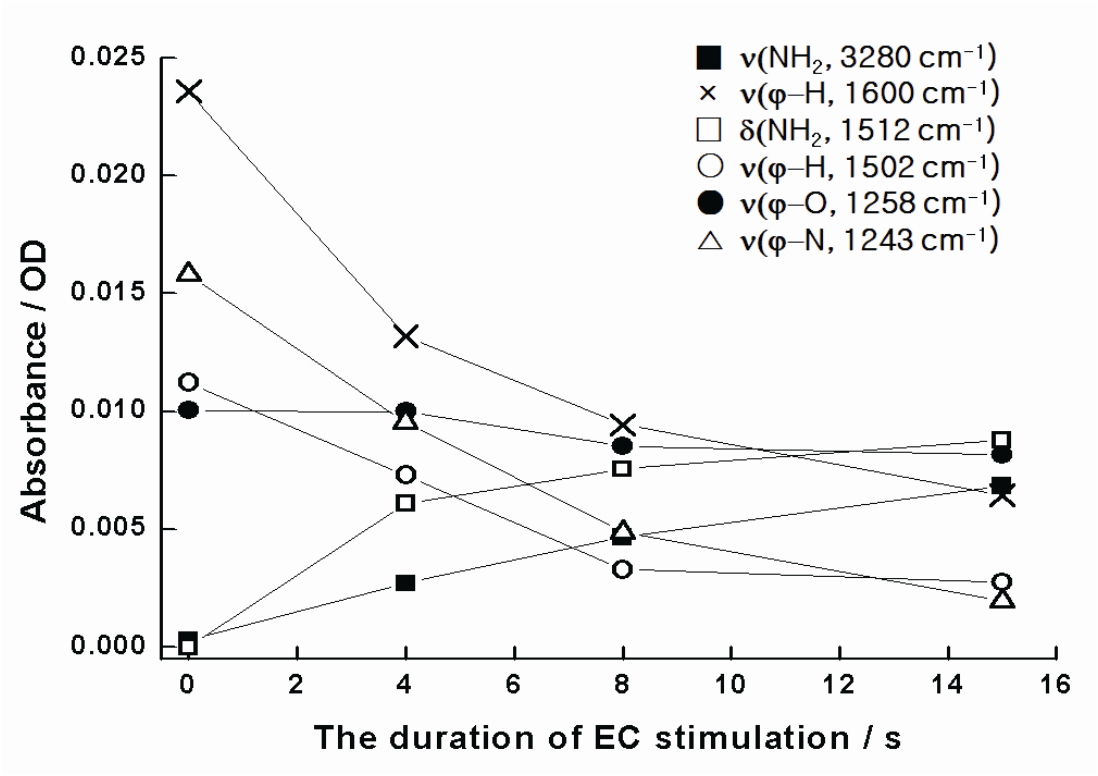

Figure S5. The variation in the absorbance of each functional group, deconvoluted from the spectra in Figure S3, as a function of the duration of electrochemical stimulation.

Table S1. Band assignments of FT-IR spectra.

Absorbance band	Vibration mode
3280 cm^{-1}	NH_2
2920 / 2850 cm^{-1}	$CH_{2,asym}$ / $CH_{2,sym}$
1603 / 1586 cm^{-1}	φ-H
1512 cm^{-1}	NH_2
1502 / 1470 cm^{-1}	φ-H
1258 cm^{-1}	φ-O
1243 cm^{-1}	φ-N

Table S2. Measured contact angles for the modified gold surfaces

Surfaces	Contact angle (deg)
Bare gold	66.3 ± 3.8
Azobenzene-terminated gold	80.2 ± 2.1
Electrochemically activated gold	52.5 ± 4.7
Aniline-terminated gold	56.5 ± 1.9