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Double Covalent Coupling Method for the Fabrication of Highly Sensitive and Reusable Electrogenerated Chemiluminescence Sensors

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A double covalent coupling method for the fabrication of a highly sensitive and reusable electrogenerated chemiluminescence (ECL) chemical sensor for the detection of tertiary amines and ECL aptamer-based (ECL-AB) biosensor for the detection of cocaine is reported. The ECL sensors were constructed by covalent coupling of amino-containing $Ru(bpy)_3^{2+}$ derivatives (Ru1, $Ru(bpy)_3^{2+}$ = tris(2,2′-bipyridyl)ruthenium(II)) or cocaine aptamer-Ru1 to the surface of a paraffin-impregnated graphite electrode that had been covalently modified with a monolayer of 4-aminobenzene sulfonic acid via electrochemical oxidations. ECL performance of the newly developed chemical sensors was evaluated using tri-*n*-propylamine (TPrA) and metoclopramide (MCP) as model analytes. The sensors exhibited excellent sensitivity, stability, and reproducibility with a detection limit of 30 nM for TPrA and 2.0 nM for MCP, and relative standard deviations (RSDs) of 2.1% over 90 cyclic potential cycles (0 to 1.50 V vs Ag/AgCl) and 2.6% over 45 cycles (0.60 to +1.30 V vs Ag/AgCl) at 400 mV/s for 50 nM TPrA and 200 nM MCP, respectively. For the ECL-AB biosensor, it showed an extremely low detection limit of 10 pM for cocaine, and offered a good selectivity toward cocaine, heroin, and caffeine. This detection limit was about 4−6 orders of magnitude lower than that reported on the basis of alternating current (AC) voltammetry and optical aptamer-based cocaine biosensors. Additionally, the ECL-AB biosensor was highly reusable (RSD = 2.8%, $n = 7$) and possessed long-term storage stability (96.8% initial ECL recovery over 21 days storage). A binding constant of $4.6 \pm 0.3 \times 10^9$ M^{-1} between cocaine and its aptamer was estimated using an ECL based Langmuir isotherm approach. Wide ranging applications of the presently reported strategy in fabricating various chemical sensors or biosensors are expected.

Electrogenerated chemiluminescence (ECL) is a method of generating light by using electrochemical reactions to produce highly reactive species at the surface of an electrode that can produce excited states in energetic electron transfer reactions.[1,2] Owing to its inherent features,[3−7] such as low background, high sensitivity, good reproducibility, and selectivity, ECL has been widely used in many chemical and biochemical related applications over the past several years, including immunoassay, DNA hybridization detection, food and water testing, as well as for biowarfare agent or explosive material detection.[3,8−18] Although many ECL systems have been well studied, the most widely used ECL system so far is the oxidative-reduction type $Ru(bpy)_3^{2+}$ (or its derivatives)/TPrA system, where $Ru(bpy)_3^{2+}$ = tris(2,2′-bipyridyl)ruthenium(II)) and TPrA = tri-*n*-propylamine, because this system has provided the highest efficient ECL emissions in aqueous solutions, and the effect of dissolved oxygen on ECL production is generally negligible.[3] Upon the anodic potential scanning, two ECL waves, located at ∼+0.90 and ∼+1.10 V versus Ag/AgCl, respectively, are observed at a Au or a glassy carbon electrode (GCE). The low potential ECL peak (1st ECL wave) is associated with the direct oxidation of TPrA and the other (2nd ECL wave) is associated with the

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(1) Liu, C.; Bard, A. J. *Nat. Mater.* **2008**, *7*, 505–509.
(2) *Electrogenerated Chemiluminescence*; Bard, A. J., Ed.; Marcel Dekker, Inc.: New York, 2004.
(3) Miao, W. *Chem. Rev.* **2008**, *108*, 2506–2553.
(4) Qi, H.; Peng, Y.; Gao, Q.; Zhang, C. *Sensors* **2009**, *9*, 674–695.
(5) Forster, R. J.; Bertoncello, P.; Keyes, T. E. *Annu. Rev. Anal. Chem.* **2009**, *2*, 359–385.
(6) Richter, M. M. *Chem. Rev.* **2004**, *104*, 3003–3036.
(7) Wei, H.; Wang, E. *TrAC, Trends Anal. Chem.* **2008**, *27*, 447–459.
(8) Debad, J. D.; Glezer, E. N.; Leland, J. K.; Sigal, G. B.; Wohlsadter, J. In *Electrogenerated Chemiluminescence*; Bard, A. J., Ed.; Marcel Dekker, Inc.: New York, 2004; Chapter 8, pp 359−396.
(9) Pittman, T. L.; Miao, W. *J. Phys. Chem. C* **2008**, *112*, 16999–17004.
(10) Pittman, T. L.; Thomson, B.; Miao, W. *Anal. Chim. Acta* **2009**, *632*, 197–202.
(11) Zhang, J.; Qi, H.; Li, Y.; Yang, J.; Gao, Q.; Zhang, C. *Anal. Chem.* **2008**, *80*, 2888–2894.
(12) Wang, S.; Milam, J.; Ohlin, A. C.; Rambaran, V. H.; Clark, E.; Ward, W.; Seymour, L.; Casey, W. H.; Holder, A. A.; Miao, W. *Anal. Chem.* **2009**, *81*, 4068–4075.
(13) Zhu, X.; Lin, Z.; Chen, L.; Qiu, B.; Chen, G. *Chem. Commun.* **2009**, 6050–6052.
(14) Li, Y.; Qi, H.; Peng, Y.; Gao, Q.; Zhang, C. *Electrochem. Commun.* **2008**, *10*, 1322–1325.
(15) Li, Y.; Qi, H.; Peng, Y.; Yang, J.; Zhang, C. *Electrochem. Commun.* **2007**, *9*, 2571–2575.
(16) Yin, X.-B.; Xin, Y.-Y.; Zhao, Y. *Anal. Chem.* **2009**, *81*, 9299–9305.
(17) Wang, X.; Dong, P.; Yun, W.; Xu, Y.; He, P.; Fang, Y. *Biosens. Bioelectron.* **2009**, *24*, 3288–3292.
(18) Yao, W.; Wang, L.; Wang, H.; Zhang, X.; Li, L. *Biosens. Bioelectron.* **2009**, *24*, 3269–3274.

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direct oxidation of $Ru(bpy)_3^{2+}$ at the electrode.[19] In other words, for the $Ru(bpy)_3^{2+}$/TPrA system, production of ECL does not necessarily need the oxidation of $Ru(bpy)_3^{2+}$ at relatively high potentials, although the second ECL wave often shows a much stronger ECL emission than the first one.

When an ECL-based detector is used in flow-injection, capillary electrophoresis, or a HPLC system for the detection and quantification of analytes such as various kinds of tertiary or secondary amine compounds that can act as an ECL coreactant, immobilization of $Ru(bpy)_3^{2+}$ or its derivatives onto a solid substrate (e.g., an electrode) is absolutely desirable.[3,20] Several advantages are offered by the surface-confined system over conventional solution-phase systems, including reducing the consumption of expensive reagent, simplifying experimental design, and creating a regenerable chemical sensor (or a chemically modified electrode). For ECL-based biosensors such an immobilization of ECL labels onto the electrode is frequently required. These biosensors combine advantages offered by the specific selectivity of the biological recognition elements (e.g., DNA/RNA strands,[11,21,22] antibodies/antigens,[23,24] DNAzyme interactions,[13] and aptamers[14−18]) and the high sensitivity of ECL transducers. As a result, extensive efforts have been made in the development of immobilization of $Ru(bpy)_3^{2+}$type complexes to the electrode via, for example, electrostatic adsorption,[25,26] physical entrapment,[27] self-assembled monolayers (SAMs),[28,29] and covalent coupling.[30,31] Although thiolated SAMs on Au for the attachment of ruthenium complexes have been broadly used, this approach can only allow for the anodic ECL measurements conducted under potentials not more positive than ~0.80 V versus Ag/AgCl so that the thiol layer's damage can be avoided.[15,18,32,33] Additionally, because gradual degradation of the SAMs in air or in a phosphate saline buffer (PBS) solution often occurs, the long-term storage stability of the ECL detector or the biosensor is a challenge.[34,35] In contrast, a covalent coupling approach could provide several attractive features which include good stability, high reproducibility, and a wider applicable potential window.

Recently, free radical grafting methods for modifying various conducting and semiconducting substrates have drawn great attention.[36−41] Wang et al. reported the ECL of stable $Ru(bpy)_3^{2+}$

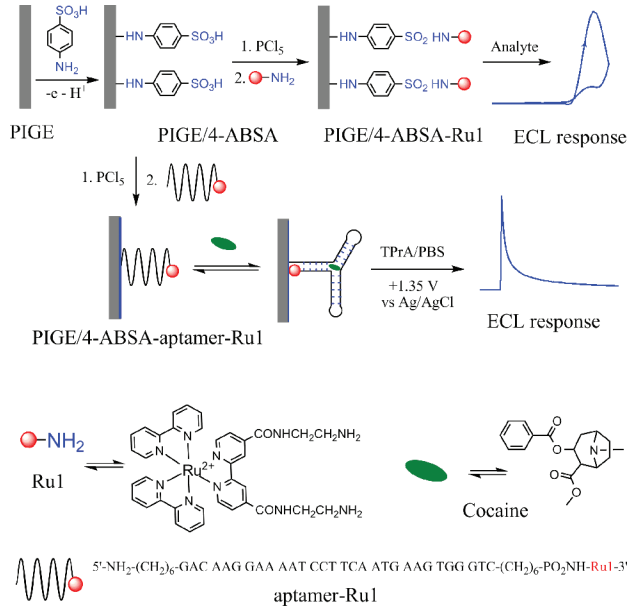

Figure 1. Schematic diagram of the fabrication of ECL chemical sensor and ECL aptamer-based biosensor for cocaine detection.

monolayer electrostatically assembled on a GCE that had been covalently bound with diazobenzene sulfonic acid via electrochemical reduction.[25] Chen et al. reported an electrochemical DNA hybridized biosensor, where the capture probe was covalently attached to a 4-aminobenzene sulfonic acid (4-ABSA) modified GCE through a terminal amine-containing DNA sequence.[42]

The aim of the present work is to develop a double covalent coupling method for the fabrication of significantly sensitive, electrochemically stable, and highly reusable ECL sensors. This will be demonstrated by a constructed ECL chemical sensor using TPrA and metoclopramide (a commonly used antiemetic and gastroprokinetic agent) as model analytes, and an ECL aptamer-based (ECL-AB) biosensor for sensitive detection of cocaine. Figure 1 illustrates the schematic diagram of the design and fabrication for the above two types of ECL sensors, which will be discussed in detail in the following sections.

EXPERIMENTAL SECTION

Reagents and Apparatus. Ruthenium bis(2,2′-bipyridine) (2,2′-bipyridine-4,4′-dicarboxylic acid)-ethylenediamine (abbreviated Ru1, Figure 1) was synthesized from ruthenium bis(2,2′-bipyridine) (2,2′-bipyridine-4,4′-dicarboxylic acid)-*N*-hydroxysuccinimide ester[43,44] and ethylenediamine as reported in our previous

(19) Miao, W.; Choi, J.-P.; Bard, A. J. *J. Am. Chem. Soc.* **2002**, *124*, 14478–14485.
(20) Danielson, N. D. In *Electrogenerated Chemiluminescence*; Bard, A. J., Ed.; Marcel Dekker, Inc.: New York, 2004; Chapter 9, pp 397−444.
(21) Miao, W.; Bard, A. *J. Anal. Chem.* **2003**, *75*, 5825–5834.
(22) Miao, W.; Bard, A. *J. Anal. Chem.* **2004**, *76*, 5379–5386.
(23) Miao, W.; Bard, A. *J. Anal. Chem.* **2004**, *76*, 7109–7113.
(24) Yin, X.-B.; Qi, B.; Sun, X.; Yang, X.; Wang, E. *Anal. Chem.* **2005**, *77*, 3525–3530.
(25) Wang, H.; Xu, G.; Dong, S. *Talanta* **2001**, *55*, 61–67.
(26) Li, J.; Xu, Y.; Wei, H.; Huo, T.; Wang, E. *Anal. Chem.* **2007**, *79*, 5439.
(27) Shen, L.; Li, X.; Qi, H.; Zhang, C. *Luminescence* **2008**, *23*, 370–375.
(28) Obeng, Y. S.; Bard, A. J. *Langmuir* **1991**, *7*, 195–200.
(29) Sato, Y.; Uosaki, K. *J. Electroanal. Chem.* **1995**, *384*, 57–66.
(30) Greenway, G. M.; Greenwood, A.; Watts, P.; Wiles, C. *Chem. Commun.* **2006**, 85–87.
(31) Tao, Y.; Lin, Z.; Chen, X.; Huang, X.; Oyama, M. *Sens. Actuators, B* **2008**, *129*, 758.
(32) Zu, Y.; Bard, A. *J. Anal. Chem.* **2001**, *73*, 3960–3964.
(33) Yang, D. F.; Al-Maznai, H.; Morin, M. *J. Phys. Chem. B* **1997**, *101*, 1158–1166.
(34) Cortes, E.; Rubert, A. A.; Benitez, G.; Carro, P.; Vela, M. E.; Salvarezza, R. C. *Langmuir* **2009**, *25*, 5661–5666.
(35) Kautz, N. A.; Fogarty, D. P.; Kandel, S. A. *Surf. Sci.* **2007**, *601*, L86–L90.
(36) Liu, J.; Cheng, L.; Liu, B.; Dong, S. *Langmuir* **2000**, *16*, 7471–7476.
(37) Harper, J. C.; Polsky, R.; Wheeler, D. R.; Brozik, S. M. *Langmuir* **2008**, *24*, 2206–2211.
(38) Harper, J. C.; Polsky, R.; Wheeler, D. R.; Dirk, S. M.; Brozik, S. M. *Langmuir* **2007**, *23*, 8285–8287.
(39) Harper, J. C.; Polsky, R.; Wheeler, D. R.; Lopez, D. M.; Arango, D. C.; Brozik, S. M. *Langmuir* **2009**, *25*, 3282–3288.
(40) Polsky, R.; Harper, J. C.; Dirk, S. M.; Arango, D. C.; Wheeler, D. R.; Brozik, S. M. *Langmuir* **2006**, *23*, 364–366.
(41) Liu, G.; Boecking, T.; Gooding, J. J. *J. Electroanal. Chem.* **2007**, *600*, 335–344.
(42) Chen, J.; Zhang, J.; Wang, K.; Lin, X.; Huang, L.; Chen, G. *Anal. Chem.* **2008**, *80*, 8028–8034.
(43) Shimidzu, T.; Iyoda, T.; Izaki, K. *J. Phys. Chem.* **1985**, *89*, 642–645.
(44) Kalyanasundaram, K.; Nazeeruddin, M. K.; Grätzel, M.; Viscardi, G.; Savarino, P.; Barni, E. *Inorg. Chim. Acta* **1992**, *198−200*, 831–839.

studies.[45,46] Imidazole, mercaptoundecanoic acid (MUA), *N*-hydroxysulfosuccinimide sodium salt (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and hexaaminеruthenium(III) chloride were purchased from Sigma-Aldrich. Phosphorus pentachloride (PCl_5) was obtained from Sinopharm Chemical Reagent Company (Shanghai, China). Tri-*n*-propylamine (TPrA), 4-aminobenzene sulfonic acid (4-ABSA), and acetone were obtained from the First Reagent Company of Shanghai (Shanghai, China). Metoclopramide (MCP) standard was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Cocaine, heroin, and caffeine were obtained from the State Narcotic Laboratory (Beijing, China). All other reagents were of analytical grade and Millipore Milli-Q water (>18 MΩ cm) was used throughout. A 30-mer cocaine aptamer adopted from the literature[47] with modified spacing linkers, 5′-NH_2-$(CH_2)_6$-GAC AAG GAA AAT CCT TCA ATG AAG TGG GTC-$(CH_2)_6$-HPO_4^--3′, was synthesized and purified with HPLC by Shenggong Bioengineering Co. Ltd. (Shanghai, China) for the preparation of ECL-AB cocaine biosensors.

ECL measurements were performed with a MPI-A ECL detector (Xi'an Remax Electronics, Xi'an, China). A commercial cylindroid glass cell was used as an ECL cell, which contained a conventional three-electrode system consisting of either a paraffin-impregnated graphite electrode (PIGE, 6.0 mm diameter with an effective electrode area of 98.9 mm^2)[48] or a PIGE modified with Ru1 or aptame-Ru1 (see below) as the working electrode, a platinum plate as the counter electrode, and an Ag/AgCl (saturated KCl) as the reference electrode, respectively. ECL emissions were detected with a photomultiplier tube (PMT) that was biased at −600 V unless otherwise stated. The UV−vis spectra were recorded using a UV−vis spectrophotometer (UV-2450, Shimadzu Corporation, Japan). The surface coverage of electroactive $Ru(bpy)_3^{2+}$ derivatives on PIGE was measured using procedures reported previously[25] with a CHI 660 electrochemical workstation (Chenhua Instruments Co., Shanghai, China).

Attachment of Ru1 to the Cocaine Aptamer (Aptamer-Ru1). The 3′ phosphate group of the cocaine aptamer was first activated by adding 200 μL of a 0.10 M imidazole solution (pH 6.80) to 2 OD (OD = "optical density"; 1 OD at 260 nm ≈ 33 μg/mL ssDNA) of the aptamer for 30 min. The formed phosphorimidazolide intermediates[49,50] were then mixed with 200 μL of 1.0 mM Ru1 complex and 100 μL of 0.20 M freshly prepared EDC, followed by incubation of the solution at room temperature for 12 h with shaking. During this process, the cocaine aptamer was covalently labeled with Ru1 through the 3′ phosphate of the aptamer and the -NH_2 group of Ru1.[49,50] The aptamer-Ru1 conjugates were subsequently separated from free Ru1 complex via centrifugation using procedures described in the literature.[46,51] Briefly, 100 μL of 3.0 M sodium acetate and 2.0 mL of cold ethanol were sequentially added to the mixed solution. The resulting solution was chilled for 24 h at −16 °C and then centrifuged for 30 min at 12,000 rpm. The precipitate (i.e., aptamer-Ru1) was washed with 200 μL of cold ethanol several times to remove any free Ru1 complex. Finally, the obtained aptamer-Ru1 was dissolved in 2 mL of 0.10 M PBS (pH 7.40) and stored at −16 °C prior to use. The concentration of aptamer-Ru1 solution was estimated to be 1.4 μM according to the value of UV−visible absorption of Ru1 at 457 nm,[15,43,44] since each aptamer was linked with one Ru1 complex (see Supporting Information for more details).

Fabrication of ECL Chemical Sensor and ECL-AB Biosensor. Two types of ECL sensors were fabricated in the present study as illustrated in Figure 1. First, the PIGE was covalently modified with a monolayer of 4-ABSA by repeated cyclic voltammetric (CV) scanning between +0.50 and +1.40 V versus Ag/AgCl in a 0.10 M KCl solution containing 5.0 mM 4-ABSA at a scan rate of 10 mV/s for four cycles[52] (see Supporting Information for more details). Second, the terminal sulfonic acid groups of the obtained electrode, designated as PIGE/4-ABSA, were activated in an acetone solution containing 40 mM PCl_5 for 30 min,[42] followed by rinsing with copious amounts of water and ethanol successively to remove any physically adsorbed materials. In the third step, the ECL chemical sensor, which was a PIGE/4-ABSA covalently attached with Ru1 complex, was fabricated by placing 5 μL of 1.0 mM Ru1 onto the surface of an activated PIGE/4-ABSA and allowing it to react for 4 h before washing with ethanol and water. This chemical sensor was designated as PIGE/4-ABSA-Ru1. For the fabrication of the ECL-AB biosensor, the activated PIGE/4-ABSA was immersed in 20 μL of 1.0 μM aptamer-Ru1 for 6 h at 37 °C and then washed thoroughly with PBS buffer and water. This biosensor had a configuration of PIGE/4-ABSA-aptamer-Ru1. Both ECL chemical sensor and ECL-AB biosensor electrodes were kept dry and stored at 4 °C in a refrigerator before use.

ECL Generation and Measurement. ECL emissions from the ECL chemical sensor were produced with CV scans between 0 and +1.50 V versus Ag/AgCl at a scan rate of 400 mV/s in 2.0 mL of 0.10 M PBS (pH 7.40) containing an appropriate concentration of TPrA or MCP. The ECL-AB biosensor was first immersed into 1.0 mL of 0.10 M PBS (pH 7.40) for 30 min to achieve thermal and ionic equilibrium and then immersed in 100 μL of a cocaine solution of interest for 4 min before washing with a PBS (pH 7.40) solution. ECL determination of cocaine was performed with a potential-step excitation from an initial potential of 0 V to a constant potential of +1.35 V versus Ag/AgCl in 2.0 mL of 0.10 M TPrA-0.10 M PBS (pH 7.40) solution. Quantification of cocaine was based on the ECL peak intensity changes, ΔI_{ECL} ($\Delta I_{ECL} = I - I_0$), where I_0 and I are the ECL peak intensity before and after the loading of cocaine, respectively.

Unless otherwise stated, all measurements were performed at room temperature (~25 ± 2 °C).

RESULTS AND DISCUSSION

Stability and Reproducibility of PIGE/4-ABSA-Ru1 ECL Chemical Sensors. Stability and reproducibility are important for any reusable chemical sensors in prospective practical applications. To demonstrate that our presently proposed approach

(45) Li, Y.; Qi, H.; Fang, F.; Zhang, C. *Talanta* **2007**, *72*, 1704–1709.
(46) Wang, H.; Zhang, C.; Li, Y.; Qi, H. *Anal. Chim. Acta* **2006**, *575*, 205–211.
(47) Baker, B. R.; Lai, R. Y.; Wood, M. S.; Doctor, E. H.; Heeger, A. J.; Plaxco, K. W. *J. Am. Chem. Soc.* **2006**, *128*, 3138–3139.
(48) Qi, H.; Zhang, Y.; Peng, Y.; Zhang, C. *Talanta* **2008**, *75*, 684–690.
(49) Chu, B. C. F.; Wahl, G. M.; Orgel, L. E. *Nucleic Acids Res.* **1983**, *11*, 6513–6529.
(50) Ghosh, S. S.; Kao, P. M.; Kwoh, D. Y. *Anal. Biochem.* **1989**, *178*, 43–51.
(51) Yang, M.; Liu, C.; Qian, K.; He, P.; Fang, Y. *Analyst* **2002**, *127*, 1267–1271.
(52) Li, X.; Wan, Y.; Sun, C. *J. Electroanal. Chem.* **2004**, *569*, 79–87.

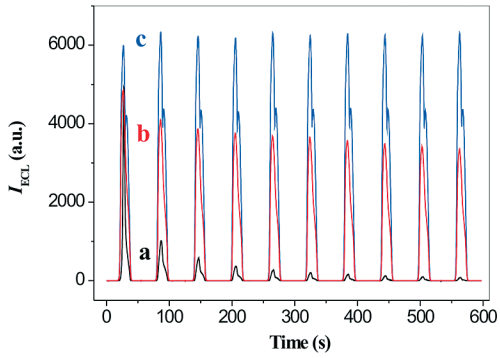

Figure 2. ECL intensity-time curves obtained from continuous potential scanning over 10 cycles between 0 and 1.50 V versus Ag/AgCl in 0.10 M TPrA-0.10 M PBS (pH 7.40) with a scan rate of 50 mV/s at different electrodes: (a) Au/MUA-Ru1, (b) PIGE/4-ABSA-Ru1 without PCl_5 activation, and (c) PIGE/4-ABSA-Ru1 with PCl_5 activation. The PMT was biased at −900 V.

is a promising method for fabricating ECL sensors, in this section, the ECL performance of the PIGE/4-ABSA-Ru1 sensor is evaluated with two other types of chemical sensors that have been reported previously.[25,28,29] The first type of sensor is constructed with a SAM of MUA thiol on Au, followed by covalent bonding of Ru1 to the SAM[28,29] (Au/MUA-Ru1, see Supporting Information section for the sensor fabrication). The second type of sensor is based on the electrostatic adsorption of Ru1 on to a PIGE/4-ABSA electrode.[25] Figure 2 shows the ECL emissions (a.u. = arbitrary units) recorded from three different ECL chemical sensors for continuous potential scanning over 10 cycles between 0 V and +1.50 V versus Ag/AgCl in 0.10 M TPrA-0.10 M PBS (pH 7.40) with a scan rate of 50 mV/s. For the Au/MUA-Ru1 sensor (Figure 2a), a decrease in ECL intensity of 79.0% in the 2nd cycle, and a 98.3% decrease in the 10th cycle, respectively, with respect to the initial ECL intensity, are observed. These significant losses of ECL signals over potential cycling can be attributed to the oxidative desorption of thiol molecules from the gold electrode at potentials more positive than ~1.0 V versus Ag/AgCl via oxidation reactions such as[53,54]

$$\text{RS-Au} + 2\text{H}_2\text{O} \rightarrow \text{Au}^0 + \text{RSO}_2^- + 4\text{H}^+ + 3\text{e} \qquad (1)$$

The above situation is remarkably improved for chemical sensors fabricated with PIGE electrodes covalently bonded with a 4-ABSA monolayer (Figures 2b and 2c). However, in this case, the stability of the chemical sensor is affected by the method for attaching the Ru1 complex to the 4-ABSA layer. When a PIGE/4-ABSA electrode is immersed into a Ru1 complex solution without pre-activation of the sulfonic acid group with PCl_5, the attachment of Ru1 molecules to the monolayer is primarily via electrostatic interactions.[25] As shown in Figure 2b, such an electrode demonstrates relatively stable ECL responses over potential cycling toward TPrA oxidation as compared with those obtained from the Au/MUA-Ru1 sensor, although 15.5% and 30.5% decreases from its initial ECL intensity in the 2nd and the 10th potential cycles are detected, respectively. The gradual loss in ECL signal is probably due to the expelling of the positively charged Ru1 molecules from the electrode surface rather than damage to the 4-ABSA monolayer during the anodic potential scanning. This explanation is supported by the data shown in Figure 2c, where nearly constant ECL signals are observed within the entire 10 potential cycles (RSD = 1.6%) for the PIGE/4-ABSA-Ru1 sensor that is fabricated through the double covalent coupling procedures as illustrated in Figure 1. Clearly, the covalent bond of C−N formed between the PIGE electrode and the 4-ABSA monolayer as well as that of (4-ABSA)SO_2−NH(Ru1) formed between the sulfonylated 4-ABSA monolayer[55] and Ru1 molecules are strong and can stand for repeated potential cycling over a range of 0 to +1.50 V versus Ag/AgCl under the present ECL experimental conditions. The stability difference of the two chemical sensors used in Figures 2b and 2c is not surprising, given the fact that the bond energy of covalent bonding has a typical value of ~100 kcal/mol whereas only 5 kcal/mol or less is for electrostatic-based binding.[56]

Close inspection of Figures 2b and 2c reveals that the average ECL intensity from the double covalent binding chemical sensor (Figure 2c) is ~1.7-fold higher than that from its electrostatic counterpart (Figure 2b). We believe that the different surface coverage of Ru1 at the electrode plays an important role in this aspect. On the basis of the integrated oxidation charge values of the respective chemical sensors in PBS solution (pH 7.40) (with the correction of background), the surface coverage of Ru1 was estimated to be 1.2×10^{-10} mol/cm^2 and 5.6×10^{-11} mol/cm^2 for double covalent binding-based chemical sensors ($n = 5$) and for electrostatic-based chemical sensors ($n = 5$), respectively. The former surface coverage is ~2.1-fold higher than that of the latter one. These data suggest that a well-packed monolayer of Ru1 was formed on the PIGE/4-ABSA-Ru1 sensor, because for a close-packed monolayer of $Ru(bpy)_3^{2+}$ the estimated surface coverage on Au is around 1.1×10^{-10} mol/cm^2.[25,57,58] Previously, a surface coverage of 6.0×10^{-11} mol/cm^2 for $Ru(bpy)_3^{2+}$ electrostatically assembled on benzene sulfonic acid modified GCE was reported,[25] which is consistent with our result (5.6×10^{-11} mol/cm^2) obtained from the electrostatic-based PIGE/4-ABSA electrode.

The stability and reproducibility of the double covalent coupling PIGE/4-ABSA-Ru1 sensor were further examined with TPrA and MCP coreactants at various concentrations over many more CV cycles. For example, when the sensor was used in a 50 nM TPrA-0.10 M PBS solution (pH 7.40), a RSD of 2.1% over 90 potential cycles was obtained (see Supporting Information, Figure S3(a)). Similarly, a RSD of 2.6% ($n = 45$) for 200 nM MCP was found (Supporting Information, Figure S3(b)). In addition, this sensor offers high sensitivities toward the detection of TPrA and MCP, which can be verified with the detection limit of 30 nM for TPrA and 2.0 nM for MCP ($S/N = 3$). The obtained detection limit for TPrA is about 1 order of magnitude lower than that obtained from $Ru(bpy)_3^{2+}$-containing composite electrodes involving function-

(53) Tencer, M.; Berini, P. *Langmuir* **2008**, *24*, 12097–12101.
(54) Widrig, C. A.; Chung, C.; Porter, M. D. *J. Electroanal. Chem.* **1991**, *310*, 335–359.
(55) Zhuang, H.-S.; Huang, J.-L.; Chen, G.-N. *Anal. Chim. Acta* **2004**, *512*, 347–353.
(56) Kim, D.; Kang, D. *Sensors* **2008**, *8*, 6605–6641.
(57) Obeng, Y. S.; Laing, M. E.; Friedli, A. C.; Yang, H. C.; Wang, D.; Thulstrup, E. W.; Bard, A. J.; Michl, J. *J. Am. Chem. Soc.* **1992**, *114*, 9943–9952.
(58) Kim, Y. T.; McCarley, R. L.; Bard, A. J. *J. Phys. Chem.* **1992**, *96*, 7416–7421.

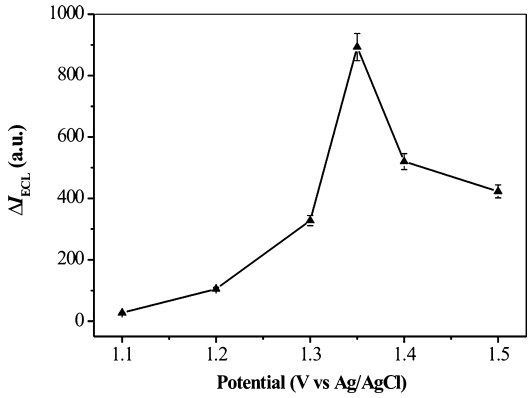

Figure 3. Effect of the working electrode potential on the ECL intensity change (ΔI_{ECL}) of the ECL-AB biosensor after interaction with 50.0 nM cocaine in 0.10 M TPrA-0.10 M PBS (pH 7.40).

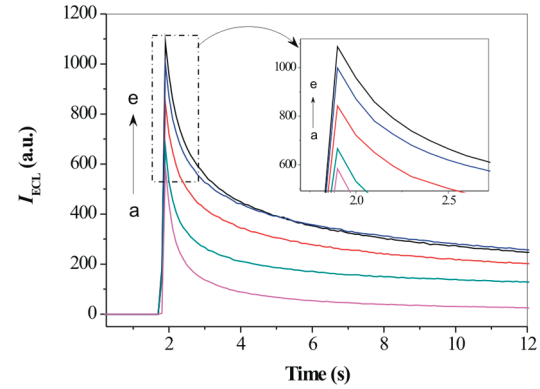

Figure 4. ECL responses of the ECL-AB biosensor placed in 0.10 M TPrA-0.10 M PBS (pH 7.40) solutions containing various concentrations of cocaine at a constant potential of +1.35 V versus Ag/AgCl: (a) 5.0×10^{-11} M, (b) 1.0×10^{-10} M, (c) 5.0×10^{-10} M, (d) 1.0×10^{-9} M, and (e) 5.0×10^{-9} M.

alized multiwall carbon nanotubes[31] and conductive polymers,[59] and 2 orders of magnitude lower than that from $Ru(bpy)_3^{2+}$ either electrostatically assembled on benzene sulfonic acid modified GCE[25] or physically entrapped in carbon paste.[27] Results also show that the ECL intensity is proportional to the TPrA and MCP concentrations over a range of 70 nM to 5.0 μM (eq 1 in the Supporting Information) and 5.0 nM to 0.50 μM (eq 2 in the Supporting Information), respectively.

ECL-AB Cocaine Biosensor. Instead of CV, a suitable single potential-step excitation for the ECL generation was found to be more sensitive toward the cocaine detection. For example, ~1.8-fold more intense in ECL peak intensity was observed from a single potential-step excitation (0 and 1.35 V vs Ag/AgCl with a pulse width of 30 s) than that from a CV sweep (0 and 1.50 V vs Ag/AgCl at a scan rate of 50 mV/s) for the ECL-AB biosensor placed in 0.10 M TPrA-0.10 M PBS (pH 7.40) containing 50 pM cocaine (see Supporting Information, Figure S4). This could be due to the simultaneous oxidations of surface-confined $Ru(bpy)_3^{2+}$ and solution-phase TPrA (used as an ECL coreactant) at the electrode (i.e., ECL-AB biosensor) that maximize the formation of the excited-state $Ru(bpy)_3^{2+}*$. The electrode was held at an initial potential of 0 V versus Ag/AgCl for 30 s before stepping up to an appropriate positive potential for 30 s. As expected, ECL emissions generated from a cocaine-loaded biosensor are strongly dependent on the step potential values (Figure 3). The potential must be positive enough to oxidize both $Ru(bpy)_3^{2+}$ (E° ~1.1 V vs Ag/AgCl) and TPrA (E_p ~0.9 V vs Ag/AgCl at a GCE)[3] without damaging the 4-ABSA monolayer and producing any undesired species from the electrolyte solution. Experimentally, +1.35 V versus Ag/AgCl was found to be the optimal potential (Figure 3). A positive potential shift of 0.25 V from the reversible redox potential of $Ru(bpy)_3^{2+}$ in solution could result from the effect of the electrode material[60,61] (i.e., PIGE vs GCE) as well as the minor electronic resistance formed between the PIGE and Ru1 redox center because of the 4-ABSA monolayer and the aptamer linkage that contains 12 $-CH_2-$ groups.[9] The decrease in ECL intensity beyond +1.35 V versus Ag/AgCl remains unclear but could be related to the oxidation of water that produces extra amounts of oxygen, resulting in the consumption of TPrA$^\bullet$ radicals needed for the generation of the $Ru(bpy)_3^{2+}*$ species.[62] Note that notable ECL emissions are always produced from the bare cocaine biosensor (i.e., PIGE/4-ABSA-aptamer-Ru1) in a 0.10 M TPrA-0.10 M PBS solution (pH 7.40) after the potential step. Thus, a change in ECL intensity (ΔI_{ECL}) resulting from the loading of cocaine is used as the analytical signal. As studied previously,[9] the production of ECL from a PIGE/4-ABSA-aptamer-Ru1 electrode with TPrA upon an anodic potential scanning/stepping can be attributed mainly to the formation of the $Ru(bpy)_3^{2+}*$ as a result of direct oxidation of TPrA. Because the Ru1 tag is linked to the PIGE electrode with a non-conductive aptamer, ECL contributions from the electrode oxidation of Ru1 should be very small, assuming that most of the aptamer-Ru1 are well tethered on the PIGE electrode as reported previously for the very similar $Ru(bpy)_3^{2+}$ attachment at an electrode surface.[9,15]

Figure 4 shows the ECL profiles of the ECL-AB biosensor after being loaded with different concentrations of cocaine. ECL emissions appear immediately after the electrode potential is stepped up to +1.35 V versus Ag/AgCl. Considerable changes in ECL peak intensity resulting from sub-pM cocaine concentration variations suggest that the present biosensor offers a very high sensitivity toward cocaine detection. Figure 5a displays the correlation between the ECL intensity changes and the cocaine concentrations. Clearly, at the relatively high concentration, intercalation of cocaine molecules into the aptamer approaches a saturated-status. Assuming the interaction process meets the requirements of the Langmuir isotherm,[63,64] the binding constant (i.e., equilibrium constant, K_b) can be expressed as:

$$\text{Aptamer-Rul}|_{PIGE} + \text{Cocaine} \rightleftharpoons \text{Aptamer-Rul}\cdot\text{Cocaine}|_{PIGE} \quad (2)$$

$$K_b = \frac{[\text{Aptamer-Rul}\cdot\text{Cocaine}|_{PIGE}]}{[\text{Aptamer-Rul}|_{PIGE}][\text{Cocaine}]} \quad (3)$$

(59) Sun, C.; Lu, W.; Gao, Y.; Li, J. *Anal. Chim. Acta* **2009**, *632*, 163–167.
(60) Wang, S.; Neshkova, M. T.; Miao, W. *Electrochim. Acta* **2008**, *53*, 7661–7667.
(61) Chen, Z.; Zu, Y. *J. Phys. Chem. C* **2008**, *112*, 16663–16667.
(62) Zheng, H.; Zu, Y. *J. Phys. Chem. B* **2005**, *109*, 12049–12053.
(63) Langmuir, I. *J. Am. Chem. Soc.* **1916**, *38*, 2221–2295.
(64) Langmuir, I. *J. Am. Chem. Soc.* **1918**, *40*, 1361–1402.

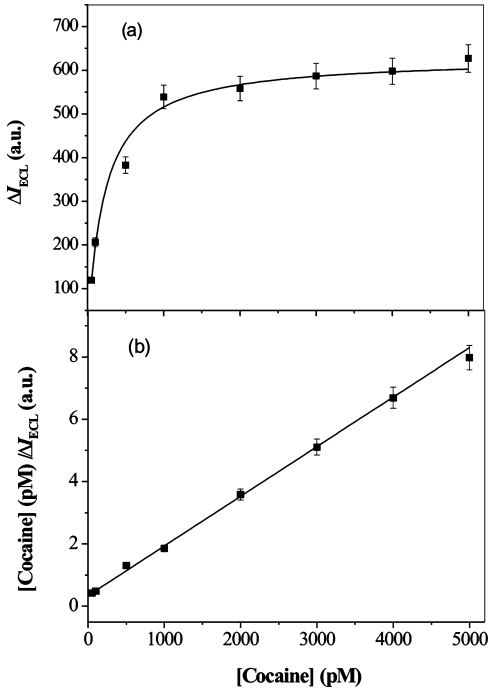

Figure 5. Langmuir isotherms obtained from the ECL-AB biosensor after interactions with various concentrations of cocaine at room temperature (~25.0 °C). (a) Non-linear regression between ΔI_{ECL} and [Cocaine], (b) linear regression between [Cocaine]/ΔI_{ECL} and [Cocaine]. See Figure 4 for experimental conditions of ECL measurements.

where [Aptamer-Ru1|$_{PIGE}$], [Cocaine], and [Aptamer-Ru1•Cocaine|$_{PIGE}$] represent the surface concentration of aptamer-Ru1 on PIGE, the solution concentration of cocaine, and the surface concentration of conjugated aptamer-Ru1•cocaine on PIGE, respectively. Thus, the relationship between the ECL intensity change (ΔI_{ECL}), the maximum ECL intensity change (ΔI_{ECL}^{max}), the solution concentration of cocaine, and the binding constant (K_b) can be described as follows:

$$\Delta I_{ECL} = \Delta I_{ECL}^{max} \frac{[\text{Cocaine}]K_b}{1 + K_b[\text{Cocaine}]} \quad (4)$$

Equation 4 can be rearranged to give eq 5:

$$\frac{[\text{Cocaine}]}{\Delta I_{ECL}} = \frac{1}{\Delta I_{ECL}^{max} K_b} + \frac{[\text{Cocaine}]}{\Delta I_{ECL}^{max}} \quad (5)$$

Therefore, [Cocaine]/ΔI_{ECL} versus [Cocaine] should give a straight line, in which $1/(\Delta I_{ECL}^{max} K_b)$ will be the intercept and $1/\Delta I_{ECL}^{max}$ will be the slope. Figure 5b depicts such a plot, which results in an intercept of 0.342 and a slope of 1.59×10^{-3} ($R^2 = 0.994$). Accordingly, $\Delta I_{ECL}^{max} = 629$ (a.u.) and $K_b = 4.6 \pm 0.3 \times 10^{-3}$ pM^{-1} (or $4.6 \pm 0.3 \times 10^{9}$ M^{-1}) are obtained. This large K_b value suggests that under present experimental conditions the binding strength between cocaine and the surface-confined aptamer is significantly strong. Other studies have shown that the binding affinities of aptamers are highly target dependent and range from the pM scale ($K_b = 1 \times 10^{12}$ M^{-1}) to the high-nM scale ($K_b = 1 \times 10^{7}$ M^{-1}) for various targets.[65−67]

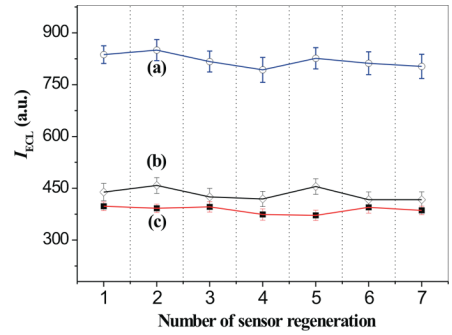

Figure 6. Examination of the ECL-AB biosensor's reusability. (a) ECL responses obtained from a 0.10 M TPrA-0.10 M PBS (pH 7.40) solution containing 5.0×10^{-10} M cocaine, (b) background ECL measured in the absence of cocaine after the cocaine-loaded biosensor was thoroughly washed with a PBS buffer solution, and (c) ECL intensity changes between (a) and (b).

A detection limit of 10 pM ($S/N \geq 3$) for cocaine was measured with our ECL-AB biosensor. This detection limit is about 4−6 orders of magnitude lower than that reported on the basis of alternating current (AC) voltammetry[47] and optical aptamer-based cocaine biosensors.[68−72] It is also about 2 orders of magnitude lower than that from our previous ECL study, where the cocaine aptamer-Ru(bpy)$_3^{2+}$ probe was self-assembled to a Au electrode through the S−Au linkage.[15] To avoid the damage of the thiol layer, a much lower positive potential of +0.80 V versus Ag/AgCl was applied.[15] Consequently, insufficient oxidation of Ru(bpy)$_3^{2+}$ as well as TPrA at the electrode resulted in a relatively high detection limit.

The selectivity of the ECL-AB biosensor was also tested using caffeine and heroin as interference compounds that belong to the narcotics family. The results showed that no significant change in ECL intensity (<5.0%) was observed from ECL-AB biosensors that had been immersed in a 0.50 nM heroin or a 0.50 nM caffeine solution, as compared with the ECL signal change generated from the same concentration of cocaine. This suggests that the present ECL-AB biosensor has an excellent selectivity for cocaine detection.

Reusability and Storage Stability of ECL-AB Biosensor. The used biosensor can be regenerated by immersing the electrode two times, 4 min each, in 2 mL of 0.10 M PBS (pH 7.40) solution at room temperature, followed by extensive washing with water. Figure 6 shows the ECL responses of an ECL-AB biosensor regenerated continuously for 7 times. The ECL signal changes (Figurer 6c) between the cocaine-loaded biosensor (Figure 6a)

(65) Nimjee, S. M.; Rusconi, C. P.; Sullenger, B. A. *Annu. Rev. Med.* **2005**, *56*, 555–583.
(66) Cho, E. J.; Lee, J.-W.; Ellington, A. D. *Annu. Rev. Anal. Chem.* **2009**, *2*, 241–264.
(67) Rusconi, C. P.; Scardino, E.; Layzer, J.; Pitoc, G. A.; Ortel, T. L.; Monroe, D.; Sullenger, B. A. *Nature* **2002**, *419*, 90–94.
(68) Stojanovic, M. N.; de Prada, P.; Landry, D. W. *J. Am. Chem. Soc.* **2000**, *122*, 11547–11548.
(69) Stojanovic, M. N.; de Prada, P.; Landry, D. W. *J. Am. Chem. Soc.* **2001**, *123*, 4928–4931.
(70) Shlyahovsky, B.; Li, D.; Weizmann, Y.; Nowarski, R.; Kotler, M.; Willner, I. *J. Am. Chem. Soc.* **2007**, *129*, 3814–3815.
(71) Liu, J.; Lu, Y. *Angew. Chem., Int. Ed.* **2006**, *45*, 90–94.
(72) Liu, J.; Mazumdar, D.; Lu, Y. *Angew. Chem., Int. Ed.* **2006**, *45*, 7955–7959.

and the unloaded one (Figure 6b) are found to have a 95% of recovery from the initial ECL intensity within the entire 7 cycles (RSD = 2.8%, $n = 7$). This finding is significant because previously reported aptamer-based ECL biosensors[14,16] could not be reused after their initial measurements because of the oxidative desorption of the thiol layers. The long-term storage stability of the biosensor was also evaluated. After being stored in a refrigerator at 4 °C for 21 and 30 days, the biosensor showed a recovery of 96.8% and 92.3% in ECL signal, respectively, where a 0.50 nM cocaine solution was used for the test. The storage stability of the present biosensor is dramatically improved (~10 days longer) as compared with a similar biosensor that we reported previously, where aptamer-$Ru(bpy)_3^{2+}$ probes were linked to a Au electrode via a thiol monolayer.[15]

CONCLUSION

A double covalent coupling method, which involves an electrochemical deposition of 4-ABSA onto a PIGE and subsequent covalent binding of an amino-containing Ru1 or an aptamer-Ru1 probe with the sulfonylated 4-ABSA monolayer, has been presented for the fabrication of ECL-based chemical sensors and aptamer cocaine biosensors. Compared with the chemical sensors constructed on a thiolated monolayer on Au or attached to the Ru1 complex electrostatically to the PIGE/4-ABSA electrode, our present chemical sensors demonstrate much higher stability, reproducibility, and sensitivity in TPrA or MCP detection. The newly reported ECL-AB biosensor has an extremely low detection limit of 10 pM for cocaine, and offers a good selectivity toward cocaine, heroin, and caffeine. This is probably due to the following two factors: (a) the large aptamer-cocaine binding constant, which has a value of $4.6 \pm 0.3 \times 10^9\ M^{-1}$ and been measured with an ECL-based Langmuir isotherm approach, and (b) the high positive potential of +1.35 V versus Ag/AgCl applied at the electrode during the ECL measurement. Additionally, the ECL-AB biosensor is highly reusable with long-term storage stability. The current work demonstrates that covalent coupling of $Ru(bpy)_3^{2+}$ derivatives and aptamer ECL probes onto the surface of PIGE/4-ABSA is a promising approach in constructing an ECL chemical sensor or an ECL-AB biosensor with high sensitivity, good stability, and significant reproducibility. The strategy could be easily extended to various analytical platforms including ECL and electrochemical biosensors as well as lab on chips for the detection and quantification of many kinds of analytes or their interactions such as DNA/RNA, DNAzyme/target, aptamer/target, and antibody/antigen.

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SUPPORTING INFORMATION AVAILABLE

Additional information is available, including the detailed fabrication of Au/MUA-Ru1 chemical sensors, UV−visible absorption spectra of aptamer, Ru1, as well as aptamer-Ru1 complex, electrochemical covalent coupling of 4-ABSA to PIGE, and examination of the stability and reproducibility of PIGE/4-ABSA-Ru1 chemical sensors. This material is available free of charge via the Internet at http://pubs.acs.org.

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