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Electrochemiluminescent chemodosimeter based on iridium(III) complex for point-of-care detection of homocysteine levels

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ABSTRACT

Elevated levels of plasma homocysteine (Hcy) are an independent risk factor for cardiovascular disease. Although a routine, rapid, and simple determination of Hcy levels is highly desired, the existing methods are practically limited because of complicated sample preparation and bulky instrumentation. Herein, we report a chemodosimetric approach for one-step analysis of Hcy levels based on the electrochemiluminescence (ECL). A rationally designed cyclometalated iridium(III) complex possessing a phenylisoquinoline main ligand underwent a selective ring-formation reaction with Hcy to generate a binding adduct, which enabled producing highly luminescent excited states, and yielded strong ECL signals on the surface of electrode without any use of enzymes or antibodies. The level of Hcy was successfully monitored by the ECL increment with a linear correlation between 0 and 40 μM in 99.9% aqueous media. The approach required neither sample preparation nor bulky instrument, suggesting the point-of-care testing of Hcy levels, and is potentially useful for routine, cost-effective, and precautionary diagnosis of various cardiovascular diseases.

1. Introduction

Homocysteine (Hcy) is a naturally occurring, sulfur-containing amino acid produced by demethylation of methionine (Wood et al., 2003; Carmel et al., 2001). It is an essential amino acid found in both animal and plant proteins. A Hcy level between 5 and 15 μM in plasma is generally considered normal (Malinow et al., 1995; Guba et al., 1996; Medina et al., 2000, 2001; Wu et al., 2002; Lievers et al., 2002). However, an elevated level of Hcy is strongly linked to cardiovascular disease (Carmel et al., 2001; Jakubowski et al., 2001; Yang et al., 2006; Nygård et al., 1997), Alzheimer's disease (Seshadri et al., 2002), neural tube defects, and osteoporosis (van Meurs et al., 2004). A previous study reported that the average Hcy level of Americans was 8 μM, and 20–30% of aged individuals (≥60 y) suffered from increased Hcy levels (> 13 μM) during 1991–2004 (Pfeiffer et al., 2008). A routine, easy-to-use procedure for the determination of Hcy is highly desirable. Various methods are now available for Hcy analysis, such as high-performance liquid chromatography (HPLC) (Uji et al., 2002; Mukai et al., 2002; Garcia et al., 2002) as well as colorimetric (Rusin et al., 2004; Wang et al., 2005; Zhang et al., 2008), radioactive (Ferguson et al., 1998; Undas et al., 2004), photoluminescence (PL) immunoassay methods (Chen et al., 2007; Zhang et al., 2007), and fluorescent molecular sensors (Lee et al., 2008, 2014; Barve et al., 2014; Peng et al., 2014). Despite showing good performance for Hcy determination, these approaches still require spectroscopic instrumentation coupled with a bulky light source. Therefore, it is highly desirable to develop a simple and easy-to-use analytical method for Hcy determination providing routine, cost-effective, and precautionary diagnosis of various cardiovascular diseases (Madasamy et al., 2015; Lee et al., 2015).

In this regard, a chemodosimetric approach coupled with electrochemiluminescence (ECL) can provide strong advantages over conventional analytical techniques; electrochemistry enables cost- and time-efficient chemical analysis in a simpler manner without requiring bulky equipment, additional reagents, or isotopes. As light emission can only be generated via an electrochemically triggered chemiluminescence process, the method is highly sensitive without background fluorescent emission (Bard et al., 2005; Richter et al., 2004). Additionally, the chemodosimetric approach would enable highly selective recognition and sensing of target molecules because of a specific, irreversible chemical reaction between a synthetic probe and target molecules (Quang et al., 2010; Yang et al., 2013). Herein, we report a new sensing strategy for qualitative and quantitative analysis of Hcy (Scheme 1) in which a cyclometalated iridium complex-based probe (**1**) undergoes selective chemical reaction with two molecules of Hcy, and the

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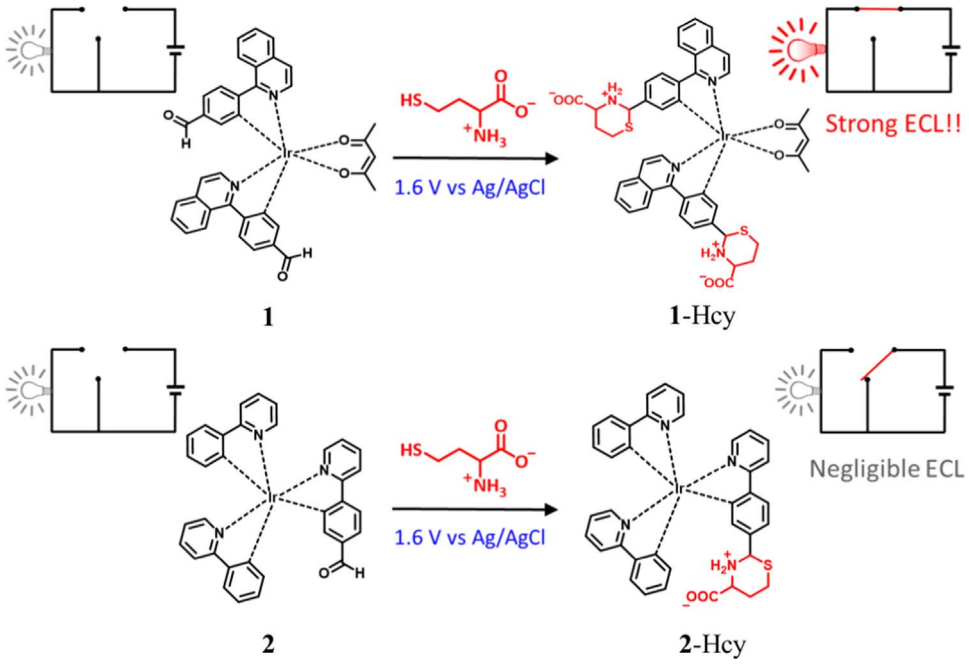


Scheme 1. ECL sensing of homocysteine in aqueous solution.

resulting adduct (**1**–Hcy) subsequently produces efficient ECL. The method is based on the following; (i) selective ring formation between a formyl group (–CHO) of **1** and Hcy, (ii) changes in the photophysical properties and energetic conditions of the probe molecule for selective target detection through the ring formation reaction, and finally (iii) the efficient ECL generated from the reaction adduct, **1**–Hcy. Probe **1** contains two aldehyde-functionalized phenylisoquinoline (piq) groups (–CHO–piq) as main ligands. The formyl group of **1** reacts with the free amine group of Hcy to form **1**–Hcy containing six-membered 1,3-thiazinanes, where sp^2 carbonyl carbons convert to sp^3 methine carbons (Fig. S1). The structural change in the main ligand caused greatly enhanced ECL of **1**–Hcy at around 615 nm, whereas **1** showed almost no ECL. We identified the thermodynamic conditions required for probe **1** to produce efficient ECL after Hcy recognition. The ECL intensity of probe **1** was gradually enhanced by selective response to the amount of Hcy. Our designed probe is able to quantify the level of Hcy with a linear correlation between 0 and 40 μM in 99.9% aqueous media. The approach provides a new proof-of-concept for point-of-care testing of Hcy levels based on ECL).

2. Experimental section

2.1. Materials and instruments

All reagents were purchased from either Sigma-Aldrich (Sigma-Aldrich Corp., MO, USA) or TCI (Tokyo Chemical Industry, Tokyo, Japan) and used without any further purification. Deuterated solvents were acquired from CIL (Cambridge Isotopic Laboratories, MA, USA). ^1H and ^{13}C NMR spectra were recorded using a Bruker Advance DPX-300. Electrospray ionization mass spectrometry (ESI-MS) data were obtained using a QUATTRO LC Triple Quadrupole Tandem Mass Spectrometer and are reported in units of mass to charge (*m/z*). High-resolution mass spectrometry (MALDI-TOF) was performed on a Voyager-DE STR Biospectrometry Workstation. Analytical thin layer chromatography was performed using Kieselgel 60 F254 plates from Merck. Column chromatography was carried out on Merck silica gel 60 (70–230 MESH) and Merck aluminium oxide 60 (70–230 MESH ASTM).

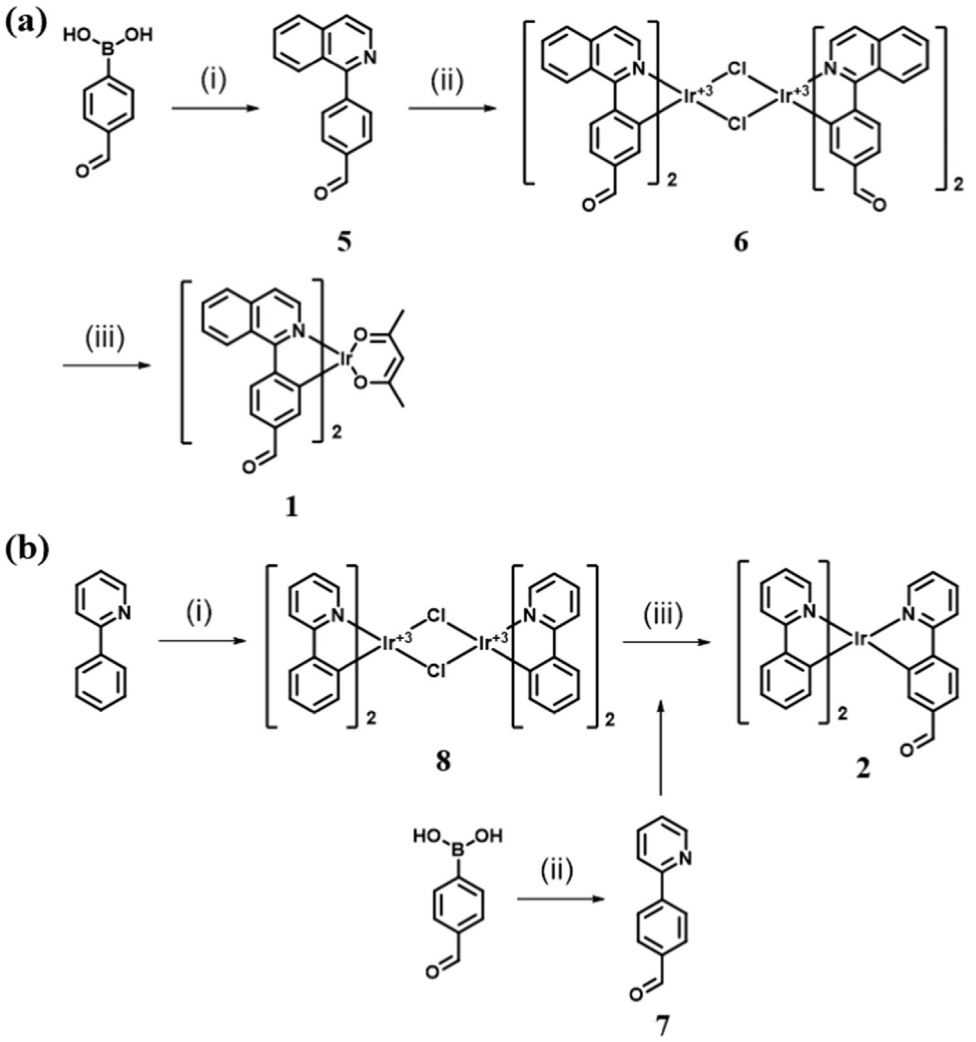
2.2. Electrochemical and electrochemiluminescent (ECL) measurements

Electrochemical study was performed with a CH Instruments 650B Electrochemical Analyzer (CH Instruments, Inc., TX, USA). Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were applied to individual solutions in order to investigate electrochemical oxidative and reductive behaviors. ECL spectra were gained using a charge-coupled device (CCD) camera (LN/CCD 1752-PB/VSAR, Princeton Instruments, NJ, USA) cooled to below −120 °C using liquid N_2. The ECL intensity profile was obtained using a low-voltage photomultiplier tube (PMT) module (H-6780, Hamamatsu photonics K. K., Tokyo, Japan) operated at 1.0 V. A 10 mL-sized ECL cell was directly mounted on the CCD or PMT module with home-made mounting support during the experiments. All the ECL data were collected via the simultaneous cyclic voltammetry on the solution. The ECL solutions commonly contained 10 mM TPA (tripropylamine, Sigma-Aldrich, MO, USA) coreactant in mixed or 99.9% aqueous solution. Especially, TPA was selected and used as an ECL coreactant as it has been widely studied and known on its electrochemical properties. The ECL measurements were carried out under ambient conditions. The electrochemical measurements were referenced with respect to an Ag/Ag^+ reference electrode in organic solvents, or to an Ag/AgCl in aqueous media. Especially, the potential values measured under organic conditions were calibrated against the saturated calomel electrode using ferrocene as an internal reference ($E_o(Fc^+/Fc)$=0.424 V vs SCE) in order to discuss electrochemical properties of the compounds (Masui et al., 1968). Pt working electrode was polished with 0.05 M alumina (Buehler, IL, USA) on a felt pad following sonication in 1:1 mixed solution of deionized water and absolute ethanol for 5 min. Then it was blown by ultra-pure N_2 gas for 1 min. A single solution was only used for one experiment, and discarded after collecting data. The reported ECL values were obtained by averaging the values from at least three or five repetitive experiments with a good reliability.

2.3. Synthesis (Scheme 2)

2.3.1. Synthesis of 4-(isoquinolin-1-yl)benzaldehyde (5)

A mixture of 4-formylphenylboronic acid (1.15 g, 7 mmol), 1-chloroisoquinoline (1.05 g, 7 mmol), $Pd(PPh_3)_4$ (404 mg, 0.35 mmol)



Scheme 2. (a) Synthesis of iridium(III) complex 1 (i) 1-chloroisoquinoline, $Pd(PPh_3)_4$, K_2CO_3, THF, H_2O, reflux; (ii) Iridium chloride hydrate, 2-ethoxyethanol, H_2O, reflux; (iii) acetylacetone, Na_2CO_3, 2-ethoxyethanol, reflux. (b) Synthesis of iridium(III) complex 2; (i) iridium chloride hydrate, 2-ethoxyethanol, H_2O, reflux; (ii) 2-bromopyridine, $Pd(PPh_3)_4$, K_2CO_3, THF, H_2O, reflux; (iii) $AgCF_3SO_3$, diglyme, 110 °C, 24 h.

and potassium carbonate (1.93 g, 14 mmol) in THF (40 mL) and H_2O (40 mL) was refluxed for 24 h. After cooling to room temperature, the reaction mixture was extracted with dichloromethane (DCM). The organic phase was washed with brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure. The residue was purified by silica gel column chromatography (ethyl acetate/hexane =1:5) to give a white solid (1.37 g, 84% yield). 300 MHz ^{1}H NMR (chloroform-d): δ 10.15 (s, 1 H), 8.65 (d, 1H, J=6.3 Hz), 8.02–8.08 (m, 3H), 7.93 (d, 1H, J=10.1 Hz), 7.89 (d, 2H, J=10.1 Hz), 7.73 (t, 1H, J=7.8 Hz), 7.72 (d, 2H, J=7.1 Hz), 7.58 (t, 1H, J=8.0 Hz).

2.3.2. Synthesis of 6

A mixture of **5** (1 g, 4.29 mmol) and iridium chloride hydrate (576 mg, 1.93 mmol) in 2-ethoxyethanol (30 mL) and H_2O (10 mL) was refluxed for 12 h. After cooling to room temperature, cold water (~50 mL) was poured into the reaction mixture. The resulting dark brown precipitate was filtered to give a crude cyclometalated Ir(III) chloro-bridged dimer (**6**).

2.3.3. Synthesis of 1

A mixture of crude cyclometalated Ir(III) dimer (6, 330 mg, 0.245 mmol), sodium carbonate (260 mg, 2.45 mmol) and acetylacetone (0.252 mL, 2.45 mmol) in 2-ethoxyethanol (10 mL) was refluxed for 8 h. After cooling to room temperature, the reaction mixture was extracted with DCM. The organic phase was washed with water and dried over Na_2SO_4. The solvent was evaporated under reduced pressure. The residue was purified by silica gel column chromatography (DCM/methanol=20:1) to give a dark brown solid (229 mg, 61% yield). 300 MHz ^{1}H NMR (DMSO-d_6): δ 9.59 (s, 2H), 9.06 (d, 2H, J=8.9 Hz), 8.46 (m, 4H), 8.24 (d, 2H, J=7.1 Hz), 7.98 (m, 6H), 7.40 (d, 2H, J=8.3 Hz), 6.70 (s, 2H), 5.28 (s, 1H), 1.70 (s, 6H). 75 MHz ^{13}C NMR ($CDCl_3$): δ 193.268, 185.179, 152.895, 151.009, 140.470, 137.315, 135.466, 134.930, 131.164, 129.581, 128.464, 127.605, 126.898, 126.500, 121.723, 121.592, 28.690. HRMS (FAB^+, m-NBA): *m/z* observed 756.1602 (calculated for $C_{37}H_{27}N_2IrO_4$ $[M]^+$756.1600).

2.3.4. Synthesis of 7 (Vandromme et al. 2008)

A mixture of 4-formylphenylboronic acid (1.15 g, 7 mmol), 2-bromopyridine (0.667 mL, 7 mmol), $Pd(PPh_3)_4$ (404 mg, 0.35 mmol) and potassium carbonate (1.93 mg, 14 mmol) in THF (40 mL) and N_2-bubbled H_2O (40 mL) was refluxed for 24 h. After cooling to room temperature, the reaction mixture was extracted with DCM. The organic phase was washed with water and dried over Na_2SO_4. The solvent was evaporated under reduced pressure. The residue was purified by silica gel column chromatography (ethyl acetate/hexane=1:5) to give a white solid (836 mg, 76% yield).

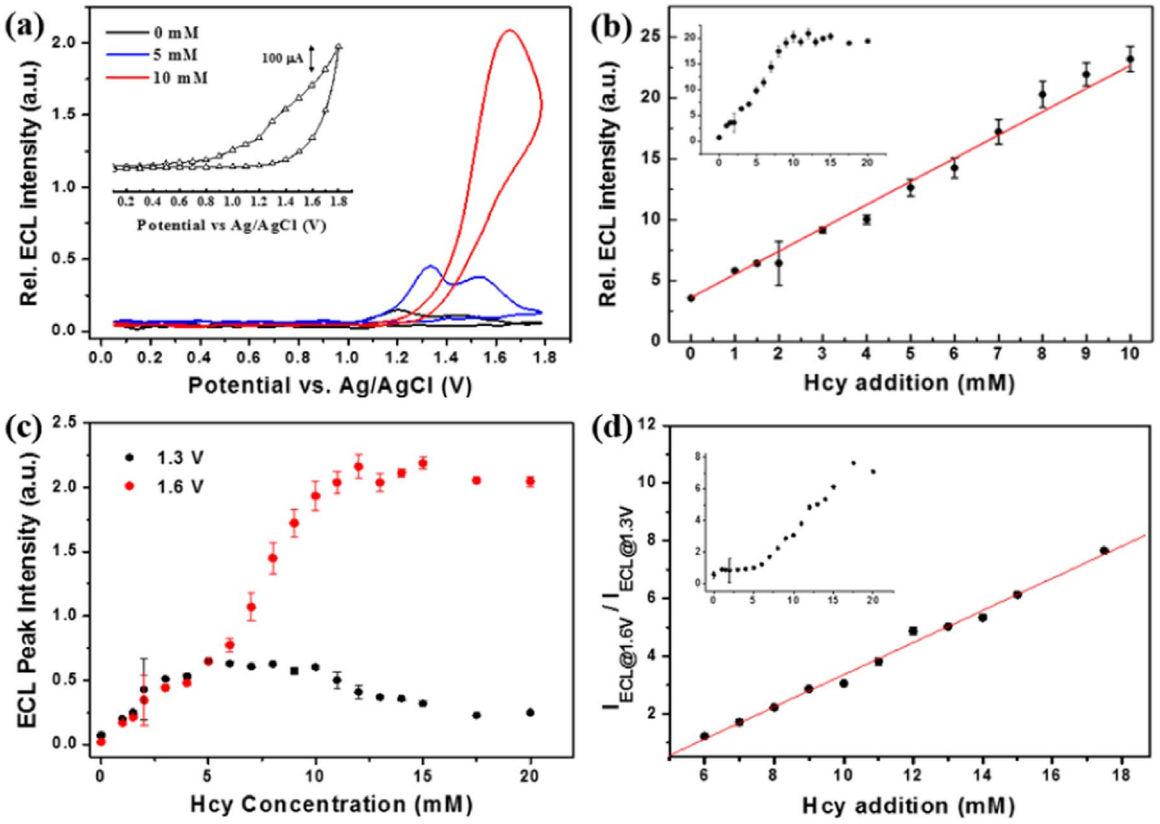


Fig. 1. (a) ECL intensity of 50 μM 1 upon addition of Hcy in H_2O/CH_3CN (1:1 v/v, pH 7.4, 10 mM TPA, 10 mM HEPES, and 0.1 M $NaClO_4$ as the supporting electrolyte) as the potential is swept at a Pt disk electrode (diameter: 2 mm) over the range 0–1.8 V vs Ag/AgCl (scan rate: 0.1 V/s). (Inset: cyclic voltammogram of a solution of 50 μM 1 in the presence of 5 mM Hcy). (b) Isothermal binding curve obtained for ECL titration upon addition of Hcy in H_2O/CH_3CN (1:1 v/v, pH 7.4, 10 mM TPA, 10 mM HEPES, and 0.1 M $NaClO_4$ as the supporting electrolyte). (c) ECL peak intensities at 1.3 and 1.6 V upon Hcy addition, and (d) ratio of the peak intensities between 1.3 and 1.6 V upon Hcy addition.

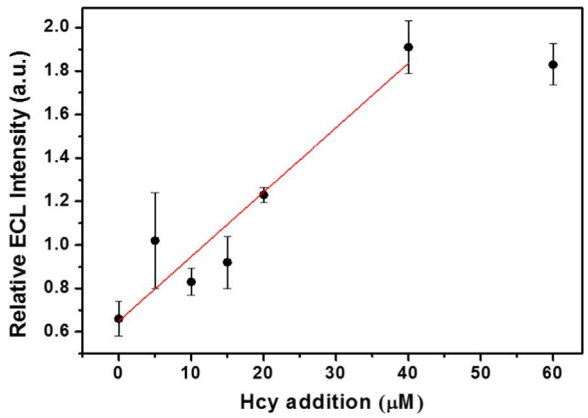


Fig. 2. ECL intensity of 0.1 μM probe 1 upon addition of Hcy in H_2O/DMSO (99.9:0.1 v/v, pH 7.4, 10 mM TPA, 10 mM HEPES, and 0.1 M $NaClO_4$).

2.3.5. Synthesis of 8 (Lamansky et al. 2001)

A mixture of **7** (1 g, 6.44 mmol) and iridium chloride hydrate (865 mg, 2.90 mmol) in 2-ethoxyethanol (45 mL) and H_2O (15 mL) was refluxed for 12 h. After cooling to room temperature, cold water (~100 mL) was poured into the reaction mixture. The resulting yellow precipitate was filtered to give a crude cyclometalated Ir(III) chloro-bridged dimer (**8**).

2.3.6. Synthesis of 2

A mixture of **7** (360 mg, 2 mmol), **8** (214 mg, 0.2 mmol), and silver trifluoromethanesulfonate (109 mg, 0.4 mmol) in diglyme (10 mL) was placed in a reaction vessel and degassed. The reaction mixture was heated at 110 °C whilst under continuous stirring for 24 h. The resulting dark orange-red solution was cooled to room temperature and water added. The suspension was filtered and the residue dissolved in DCM. The solution was then purified by silica gel column chromatography to furnish a red solid (20% yield). 300 MHz ^{1}H NMR ($CDCl_3$): δ 9.71 (s, 1H), 7.99 (d, 1H, J=4.4 Hz), 7.92 (d, 2H, J=4.4 Hz), 7.77 (d, 1H, J=8.7 Hz), 7.69 (m, 6H), 7.51 (d, 1H, J=8.4 Hz), 7.48 (d, 1H, J=8.7 Hz), 7.31 (d, 1H, J=9.5 Hz), 7.01 (t, 2H, J=6.7 Hz), 6.96 (m, 7H), 6.79(d, 1H, J=7.4 Hz). 75 MHz ^{13}C NMR (CDCl3): δ 194.603, 166.658, 166.593, 165.331, 161.738, 160.245, 159.804, 149.961, 147.505, 147.070, 146.960, 145.799, 143.679, 143.524, 141.489, 137.166, 137.027, 136.469, 136.270, 136.191, 130.925, 130.202. 129.979, 128.840, 124.119, 124.014, 123.310, 122.053, 121.975, 120.330, 120.102, 120.065, 118.966, 118.920. HRMS (FAB^+, m-NBA): *m/z* observed 683.1538 (calculated for $C_{37}H_{27}N_2IrO_4$ $[M]^+$683.1548).

2.4. Design of ECL probe 1

We selected highly luminescent $(piq)_2Ir(acac)$ (piq=1-phenylisoquinoline; acac=acetylacetonate, Φ_{PL}=0.2, λ_{max}=613 nm) as a model compound (Fig. S2) for **1**–Hcy. It showed almost Nernstian oxidation at an easily attainable potential ($E_{o'ox}$=0.74 V vs SCE) and exhibited efficient orange-red ECL at around 615 nm with a tri-*n*-propylamine (TPA) coreactant under air-saturated conditions. The relative intensity of ECL emission from $(piq)_2Ir(acac)$/TPA was estimated to be 2 times higher than that from the electrochemical reaction between $Ru(bpy)_3^{2+}$ and TPA (Fig. S2). Compared to $(piq)_2Ir(acac)$, probe **1**, which possesses an additional formyl group at the 4-position of the phenyl ring of each piq ligand, showed dramatically decreased PL (Φ_{PL}=~0.001) in the red-shifted region (λ_{max}=655 nm) due to the strong electron withdrawing ability of the formyl groups. However,

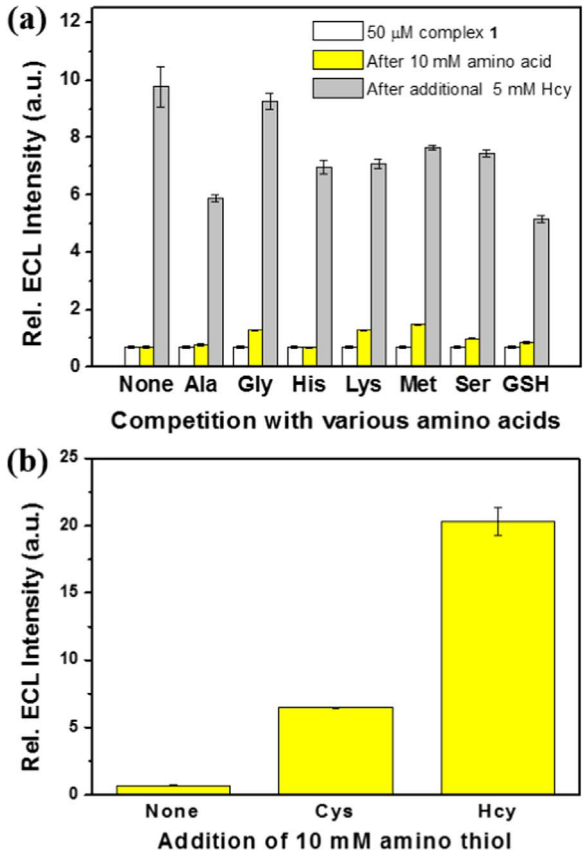


Fig. 3. (a) Competitive ECL binding assays performed with addition of 5 mM Hcy to 50 μM probe 1 in the presence of 10 mM various amino acids. (b) Comparison of ECL intensity after addition of 10 mM Hcy and 10 mM Cys to 50 μM probe 1 in H_2O/CH_3CN (1:1 v/v, pH 7.4, 10 mM TPA, 10 mM HEPES, and 0.1 M $NaClO_4$).

largely enhanced PL (Φ_{PL}=0.11), similar to that from $(piq)_2Ir(acac)$, was recovered owing to the disappearance of the formyl groups when excess Hcy was added to the solution of **1** (λ_{max}=613 nm) (Figs. S3 and S4). This blue-shifted emission is related to the increase in the HOMO-LUMO energy gap of the organometallic complex; the loss of formyl groups via reaction with Hcy caused the LUMO level of the binding adduct to increase (Figs. S6 and S7).

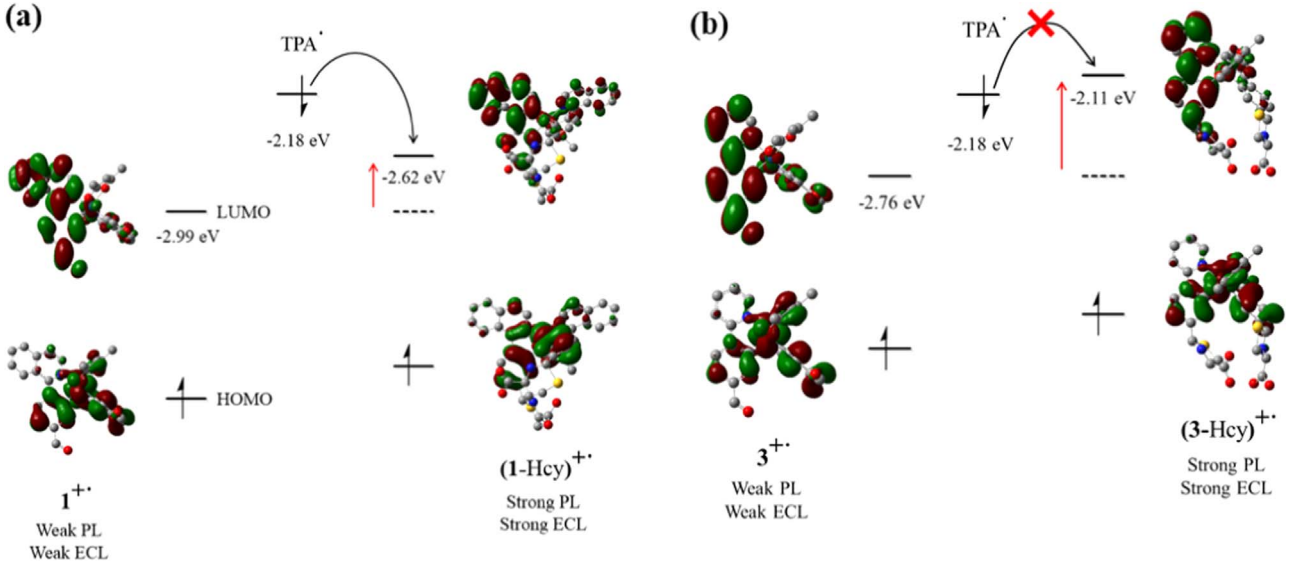
3. Results and discussion

3.1. ECL properties of 1

ECL measurements were performed in an aqueous mixture of 50 μM probe **1** and 10 mM TPA. During the cyclic voltammetry (CV) process, no significant ECL was observed from **1** in aqueous media (pH 7.4, HEPES buffer/CH_3CN=1:1 v/v), but the emission intensity gradually increased with addition of Hcy. The ECL emission profile of the probe solution is shown in Fig. 1a; while no emission was observed in the absence of Hcy, ECL showed two maximum intensities at around 1.3 and 1.6 V (vs Ag/AgCl) upon addition of 5 mM Hcy, and a single-spiked, enhanced emission peak was observed at 1.6 V when 10 mM Hcy was added to the solution. A binding titration curve was obtained from plots of the ECL intensity of **1** against various concentrations of Hcy (Fig. 1b). This indicates that the ECL signal increases linearly with Hcy addition over the range of 0–10 mM Hcy when the concentration of **1** is kept constant at 50 μM (R^2=0.995, y=2.0×10^{-9}x+1.7×10^{-9}). The estimated limit of detection (LOD) was 41 μM (signal-to-noise (S/N) ratio=3, n=5).

Interestingly, as shown in Fig. 1a, the two emission peaks appear in the ECL titration profiles; during the first stage of Hcy addition, the two ECL emission peaks at 1.3 and 1.6 V gradually increase until 5 mM (100 equiv.) Hcy was added. Upon further addition of Hcy, the former peak decreases whereas the latter continues to increase. Fig. 1c shows the variation in ECL peak intensity at 1.3 and 1.6 V. The signals at 1.3 and 1.6 V increase until addition of 100 equiv. Hcy (5 mM). Then, a new increment starts at 1.6 V, reaching saturation at 200 equiv. Hcy (10 mM), while the ECL intensity at 1.3 V decreases. Since **1** contains two formyl groups, it was expected to react with Hcy in a 1:1 M ratio in the early stages of Hcy addition, followed by the formation of 1:2 adduct **1**–Hcy in the later stages. So, the increase around 1.3 V is presumably due to the formation of a 1:1 product of **1** and Hcy, and the signal at 1.6 V originates from the final 1:2 product (**1**–Hcy). A ratiometric approach exploiting the two signals at 1.3 and 1.6 V provides a linear calibration curve over the range of 6–17 mM Hcy (Fig. 1d, R^2=0.996, y=0.56x–2.2).

Blood Hcy levels are normally between 5–15 μM and approximately 5 μM Hcy should therefore be detectable in order to assess potential risks to patients. Our estimated LOD (41 μM) is higher than that required for practical determination of Hcy abnormalities (Wood et al., 2003; Carmel et al., 2001; Yang et al., 2006). Thus, we fabricated a 25 μL volume ECL cell, modified from our previous flow cell system, in



Scheme 3. Thermodynamic explanation for Hcy sensing of probes **1** and **3** obtained by DFT calculations.

order to lower the detection limit for Hcy (Shin et al., 2011). The cell was placed directly in front of a photon multiplier tube detector with a short path length of 1 mm, so that higher sensitivity for ECL emission was expected. Using the new system, 0.1 μM **1** was incubated with various amounts of Hcy in 99.9% aqueous media, and finally a linear correlation between the ECL intensities and Hcy concentrations was obtained over the range 0–40 μM ($y=(3.0\times10^{-2})x+6.5\times10^{-1}$), which almost covers the required range for clinical diagnosis (Fig. 2). The sensitivity of **1** towards Hcy is almost comparable to that in previous reports, most of which showed a dynamic range from milimolar to micromolar levels (Chen et al., 2013; Barve et al., 2014; Lee et al., 2014; Peng et al., 2014). The LOD of Hcy was estimated to be 7.9 μM (S/N ratio=3, n=5).

3.2. Selectivity test

A competitive binding assay was carried out to evaluate the selectivity of **1** for Hcy recognition in the presence of other amino acids. 5 mM Hcy was added to a solution of 50 μM **1** containing 10 mM other amino acids or glutathione (GSH) (Fig. 3a). The results show that the ECL intensity of **1** changes slightly upon addition of a 200-fold excess of other amino acids. However, a remarkable change in the ECL intensity occurs when 5 mM (100 equiv.) Hcy is added to a solution of **1** and amino acids; despite the presence of a 200-fold excess of amino acids, the emission intensity approached 54–97% of that obtained in the absence of amino acids. PL competition assays showed similar results (Fig. S8), indicating that **1** selectively recognizes Hcy without interference from other amino acids.

Only cysteine (Cys) can cause strong interference because of its structural similarity to Hcy. As shown in Fig. 3b, an ECL signal was also observed upon addition of 10 mM Cys, indicating that **1** formed a binding adduct with Cys as well as with Hcy. However, **1** shows 3.2 times stronger ECL intensity with Hcy than with Cys. These results correlate well with our previous findings, where a CHO-containing fluorescent probe showed 2.5 times higher selectivity to Hcy compared to Cys in fluorescence binding assays (Lee et al., 2008).

3.3. Comparison with other Ir(III)-based probes (2, 3)

PL determination of Hcy with similar probes has been previously reported by us and other groups (Lee et al., 2008; Chen et al., 2007; Zhang et al., 2007; Chen et al., 2011). Huang et al. developed a –CHO-containing Ir(III) complex, $(pba)_2Ir(acac)$ (**3**, pba=4-(2-pyridyl)benzaldehyde), which exhibited enhanced phosphorescence intensity with blue-shifted emission upon Hcy recognition (Chen et al., 2007). Recently, Schmittel group reported a similar approach, where enhanced PL intensity was achieved upon reaction of an Ir(III) complex, $(ppy)_2Ir(FIMP)$ (FIMP=2-(4-formylphenyl)imidazo[4,5-f][1,10]phenanthroline) with Hcy, but almost negligible ECL change was observed after target recognition (Chen et al., 2013).

From an electrochemical viewpoint, destabilization of the LUMO distribution of the probe as a result of –CHO breakdown causes its reduction potential to shift to a more negative value. Therefore, in the previous report, (Chen et al., 2007) the binding adduct of $Ir(pba)_2(acac)$ (**3**) and Hcy was expected to possess a cons iderably negative reduction potential. In the ECL process, however, the highly negative reduction potential prevented electron transfer from the TPA radical to the LUMO of the binding adduct, hardly generating the excited states (Shin et al., 2010, 2007; Kim et al., 2005). Iridium complexes possessing 2-phenylpyridine ligands are known to have slightly more negative reduction potentials than the TPA radical, (Kim et al., 2005; Tsuzuki et al., 2007; Barbanate et al., 2014) and therefore, elevation of the LUMO of **3** after reaction with Hcy would presumably quench its ECL by preventing energy transfer from the TPA radical to Ir(III) complex (Scheme 3b).

Taking this into consideration, we rationally designed Ir(III) complex (**1**) as an efficient ECL probe for Hcy determination. In other words, we changed pyridine to a more strongly electron withdrawing isoquinoline unit in the main ligand for stabilization of the LUMO, which would result in a turn-on ECL signal of **1**–Hcy, as in the PL experiments.

Density functional theory (DFT) calculations predicted that the LUMO energy level of **1**–Hcy is lower than that of the HOMO of the TPA radical, even after formation of the adduct **1**–Hcy, so that **1** is expected to generate efficient ECL in response to Hcy (Scheme 3a). On the other hand, the LUMO energy level of **3**, the probe used in Huang's report, is drastically elevated after reaction with Hcy and is consequently higher than the HOMO energy level of the TPA radical (Scheme 3b). In this case, ECL generation could not proceed properly because electron transfer from the TPA radical to the LUMO of $(\mathbf{3}\text{–Hcy})^{+\cdot}$ would be unfavorable.

In order to confirm this experimentally, we synthesized two iridium complexes, $(pba)_2Ir(acac)$ (**3**) and $(ppy)_2Ir(pba)$ (probe **2**, Scheme 1). The former is that reported by Huang et al. and the latter is a new Ir(III) complex possessing almost the same physicochemical properties as **3.** **2**–Hcy, the product of reaction between **2** and Hcy, showed largely enhanced PL intensity with a significant blue-shift, similar to that of **3** (Fig. S9). As expected, the ECL intensity was gradually attenuated upon increasing addition of Hcy to **2** (Fig. S10); the reaction mixture of 50 μM **2** and 10 mM Hcy showed further decreased ECL during CV (between 0 and 1.5 V at 0.1 V/s), while **2** showed weak ECL around 1.2 V before Hcy addition (Fig. S10). The ECL properties of **3** and **3**–Hcy also showed similar tendencies to those of **2** and **2**–Hcy, respectively (Fig. S11). However, probe **1** exhibited bright PL and ECL images upon treatment with Hcy (Fig. S12).

The CV studies further supported the theoretical predictions. As shown in Table S1, the reduction potential of $(ppy)_2Ir(pba)$ (**2**) is $E^{o'}_{red}$=−1.66 V (vs SCE), which negatively shifts to −1.99 V after reaction with Hcy. The reduction potential of the TPA radical is estimated as ~−1.7 V, (Lai et al., 2003) which provides an explanation as to why the reaction adduct **2**–Hcy hardly produces the excited states via the ECL process. However, in the case of probe **1**, the reaction adduct **1**–Hcy has a relatively mild reduction potential (E^{o}_{red}=−1.28 V), which is less negative than the reduction potential of the TPA radical (−1.7 V). Therefore, efficient production of the excited states and ECL emission are expected.

4. Conclusion

We demonstrated the first example of ECL-based chemodosimetric Hcy analysis involving exclusive turn-on ECL emission after selective reaction with Hcy. In the presence of Hcy, the synthetic probe **1** exhibits strong light emission in aqueous solution by electrochemical triggering. The observed ECL upon Hcy addition is distinguishable from that upon addition of Cys, GSH, and other amino acids. Monitoring the emission profile against the applied potential provides additional mechanistic information on the reaction between **1** and Hcy which cannot be obtained in a typical PL study. We expect that our systematic chemodosimetric approach will emerge as a useful tool for developing various ECL probes based on Ir(III) complexes for small biotargets.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2017.01.010.

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