

Design and Application of Multi-functional Electrogenerated Chemiluminescence Imaging Analyzer

Guangfu JIANG,* Xia LIU,* Yaqin WANG,* Sanpeng RUAN,* Honglan QI,* Yong YANG,**
Qishe ZHOU,** and Chengxiao ZHANG*†

* Key Laboratory of Analytical Chemistry for Life Science of Shaanxi Province, School of Chemistry and Chemical Engineering, Shaanxi Normal University, Xi'an, 710062, P. R. China

** Xi'an Remax Analysis Instruments Co. Ltd., Xi'an, 710075, P. R. China

A multi-functional electrogenerated chemiluminescence (ECL) imaging analyzer including both a photomultiplier tube and charged coupled device as detectors has been developed. The ECL imaging analyzer can effectively work for electrochemical study, ECL intensity detection at electrode array, and ECL imaging at bipolar electrodes or electrode array. As an ECL imaging example, an ECL biosensor for visual detection of matrix metalloproteinase 7 in the range from 0.05 to 1 ng/mL is demonstrated.

Keywords Electrogenerated chemiluminescence, imaging analyzer, matrix metalloproteinase 7

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Introduction

Electrogenerated chemiluminescence (ECL) is the process in which electrogenerated radicals form excited species that emit light without the need for an external light source.¹⁻⁵ The ECL method possesses several advantages over chemiluminescence and fluorescence methods since ECL is easily controlled in terms of the time and position of the light-emitting reaction and does not involve a light source, eliminating the attendant problems of scattered light and luminescent impurities. It has proven to be a very effective analytical method for its high sensitivity, rapidity and easy controllability, leading to the development of various ECL sensing approaches for immunosensing,⁶ DNA hybridization assays⁷ and enzymatic biosensors.⁸ It has also been widely used in flow injection analysis, high-performance liquid chromatography, capillary electrophoresis, and micro total analysis systems.^{9,10} The basic components of an ECL analyzer include an electrical energy supply for the ECL reaction at an electrode within an electrochemical cell and an optical detector for the measurement of either the emitted light intensity (as photocurrent or counts/s) or its spectroscopic response. The first commercial ECL instrument was produced by IGEN International, Inc. (now BioVeris, part of Roche Diagnostics Corp.) in 1994, which was adapted to measure ECL labels [Ru(bpy)₃²⁺ or its derivatives] present on the surface of magnetically responsive beads in the presence of tripropylamine (TPA).^{11,12} After that, in the past two decades, certain types of ECL analyzers become commercially available, such as, The Sector Imager and Sector PR Reader ECL instruments, MESO QuickPlex SQ 120 and SECTOR PR 400/100 instruments developed by Meso Scale Discovery

(Maryland, USA);¹³ a commercial CE-ECL system by Xi'an Remax Electronic Co. Ltd. (Xi'an, China);¹⁴ and M-series ECL analyzers developed by BioVeris Corp. (Maryland, USA).¹⁵ A number of strategic alliances between IGEN and companies such as Boehringer Mannheim (Mannheim, Germany), Eisai (Toyko, Japan) and Organon Teknika (Oss, The Netherlands) also promise to develop new analytical tools based on ECL technology. Although these systems function well in all standard immunometric assays tested to date, the system in enzyme-cleavage assay is seldom reported.

Among these ECL instruments, two types of detectors are usually used, including a photomultiplier tube (PMT) and charged coupled device (CCD). PMTs usually provide the most sensitive means of detecting light and are capable of detecting single photons. However, ECL analyzers constructed on the basis of a PMT as a light detector can only provide ECL intensity information in one working electrode/biosensor. CCD can provide not only intensity-based quantitative information at a single electrode or electrode array, but also the qualitative information including color and shape on electrode array. It has been used in the characterization of electrode reactive surfaces,¹⁶⁻¹⁸ investigation of flow liquid dynamics,¹⁹ analysis of DNA damage,²⁰ multi-analyte immunoassays,^{21,22} and cell assay.²³ Although much improvement has been achieved with ECL imaging, most of the ECL studies reported in the literature were carried out with homemade ECL imaging instruments. A multi-functional ECL imaging analyzer including a PMT and CCD as detectors has not been reported so far.

The aim of this work is to design and construct a multi-functional ECL imaging analyzer including both a PMT and CCD as detectors. In this paper, we present a new multi-functional ECL imaging analyzer and its analytical applications in biosensing. Details of the design and construction of the ECL imaging analyzer, including hardware and software, are presented. In addition, two types of the ECL array system,

† To whom correspondence should be addressed.

E-mail: honglanqi@snnu.edu.cn; cxzhang@snnu.edu.cn

including screen-printed carbon electrodes (SPCEs) and bipolar electrodes, were fabricated to get multiple ECL signals. As an example, an ECL imaging biosensor is developed to monitor matrix metalloproteinase 7 (MMP-7) using the ECL imaging analyzer.

Experimental

Reagents and chemicals

Tris(2,2'-bipyridine) dichlororuthenium(II) hexahydrate ($\text{Ru}(\text{bpy})_3^{2+}$), bis(2,2'-bipyridine)-4'-methyl-4-carboxybipyridine-ruthenium *N*-succinimidyl ester-bis(hexafluorophosphate) ($\text{Ru}(\text{bpy})_2(\text{mcbpy-O-Su-ester})(\text{PF}_6)_2$, abbreviated as Ru1), 6-mercapto-1-hexanol (MCH), matrix metalloproteinase-7 human (recombinant, expressed in *E. coli*, 28 kDa) and Brij® L23 were purchased from Sigma-Aldrich (USA). A specific peptide KRPLALWRSC (MW = 1229.52) was obtained from Shanghai Apeptide Co., Ltd. (Shanghai, China). Ethylene-vinyl acetate copolymer was obtained from Shanghai Microspectrum Chemical Technology Service Co., Ltd. (Shanghai, China). Tripropylamine (TPA) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All chemical reagents were of analytical grade and used as received, and Millipore Milli-Q water (18.2 M Ω ·cm) was used. The 0.1 M carbonate buffer consisted of 0.10 M Na_2CO_3 and 0.10 M NaHCO_3 (pH 8.5). The 10 mM phosphate buffer (PB, pH 7.4) contained 10 mM NaH_2PO_4 and 10 mM Na_2HPO_4 was used as washing solution.

Fabrication of electrode array

A base electrode array consisting of four screen-printed carbon working electrodes, one screen-printed carbon auxiliary electrode and one Ag/AgCl reference electrode were fabricated by screen-printing technology according to the reference.²⁴ A schematic diagram of the preparation processes of the SPCE system in this study is shown in Fig. S1 (Supporting Information). An electrochemical cell (ECL cell, 20 × 12 mm, 2 mm thick, 600 μL) was designed by using ethylene-vinyl acetate copolymer as the insulating dielectric material. A 2 × 3 bipolar gold electrode was fabricated according to the reference²⁵ and the preparation process of the bipolar electrode system in this study is shown in Fig. S2 (Supporting Information).

Fabrication of ECL biosensor and bioassay of MMP-7

The Ru1-labeled peptide (Ru1-peptide) was synthesized by covalently labeling peptide KRPLALWRSC with Ru1 and then purified by dialysis using MD36-1Da molecular (1000) weight cutoff membrane with 10 mM PB (pH 7.4).

The procedure for the deposition of gold nanoparticles (GNPs) on SPCEs was adapted from literature procedures.²⁶ The ECL biosensor was fabricated by drop-coating 20 μL of 10 μM Ru1-peptide solution onto the surface of the desired GNPs-modified SPCEs and incubating for 1 h at room temperature. And then the resulting electrode was washed with the washing buffer and was subsequently blocked with 1.0 mM MCH for 30 min.

Next, 200 μL of TCNB buffer (50 mM Tris with 10 mM CaCl_2 , 150 mM NaCl , and 0.05% Brij 35; pH 7.5) containing a certain amount of MMP-7 was dropped onto the ECL biosensor for 1 h at 37°C and then the cleaved biosensor was washed with 10 mM PB. After 0.6 mL of 0.10 M PBS (10 mM NaH_2PO_4 , 10 mM Na_2HPO_4 and 0.1 M KCl , pH 7.4) contained 50 mM TPA was injected to the ECL cell, the ECL measurement was performed using the ECL imaging analyzer in two models. In

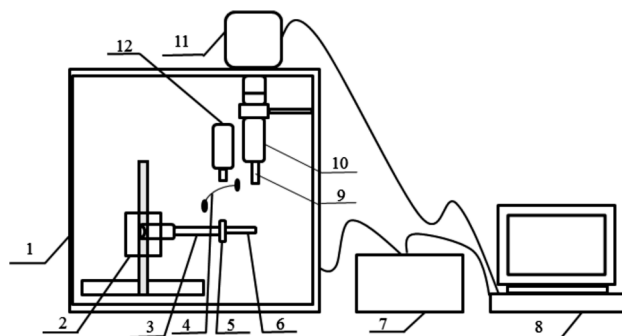


Fig. 1 Scheme of the hardware of the ECL imaging analyzer. 1, Dark box; 2, XYZ electric stage; 3, adjustable DC voltage and potentiostat; 4, LED light; 5, electrode cell connector; 6, ECL cell; 7, main control; 8, computer; 9, microobjective; 10, optical module; 11, EMCCD; 12, PMT.

the PMT detection model, the ECL measurement was performed using a triangular potential scan with the rate of 0.1 V/s. In the electron-multiplying CCD (EMCCD) detection model, the ECL measurement was performed at a fixed potential with a fixed exposure time. The value of ECL counts in EMCCD images was analyzed using in-house ImageJ software that is specifically designed for this system, written by Xi'an Remax Analysis Instruments Co. Ltd. (China). The concentration of MMP-7 was quantified by a decreased ECL count ($\Delta A = A_0 - A_s$), where A_s and A_0 were the ECL counts of the ECL biosensor after and before reaction with MMP-7, respectively.

Results and Discussion

Design of the ECL imaging analyzer

The components of the multi-functional ECL imaging analyzer include the hardware and the software. The design ideal and basic functions are presented in the following sections.

The hardware

The hardware of the ECL imaging analyzer was designed to consist of an electrochemical part, light detection part and automation movement platform part (as shown in Fig. S3, Supporting Information). The scheme of the hardware of the ECL imaging analyzer is showed in Fig. 1.

Electrochemical part. The functions of this part are to provide the applied potentials for electrochemical detection and the required potentials for ECL emission detection using a PMT or EMCCD. The hardware of this part includes adjustable DC voltage supply and potentiostat, including a 0 – 150 kV DC power supply for providing the voltage for bipolar electrodes and a ± 10 V potentiostat for supplying the voltage for SPCEs.

Light detection part. The ECL emissions generated on the desired working electrode are collected with either an ultralow-noise EMCCD camera for fast and multiple imaging or a PMT for ultimate sensitivity detection. The EMCCD imaging model and PMT intensity model are designed to perform separately. In the ECL imaging model, the main body of an EMCCD is placed outside the black box to be cooled effectively while the window of an EMCCD is directly pointed to the working electrodes. More importantly, a series of micro-objectives (4 $\times$, 10 $\times$, 20 $\times$ and 40 $\times$), and optical lenses are designed to effectively transfer the ECL emission. In the PMT model, the window of a PMT is directly pointed to the working electrodes.

Automation movement platform part. For the most precise control of the distance of PMT/EMCCD-to-electrode, a mobile stage was incorporated into the system's design. A stage is positioned for moving scanning in the X , Y and Z dimensions over a range of 50×50 mm with a $2 \mu\text{m}/\text{Step}$ precision in X and Y dimensions and $10 \mu\text{m}/\text{Step}$ precision in Z dimensions controlled by a personal computer. The ECL cell was inserted to the connector like a USB inserter. The whole ECL detection system was held in a light-tight black box constructed from black materials to create a darkroom. Additionally, an LED light was used in order to take a picture in the bright field measurement and see easily the position of the electrodes in the black box.

The software

The software developed for the ECL imaging analyzer is controlled by an in-house software program that is specifically designed for this system. The software allows the user to control all aspects of the instrument enabling complete automated control of all components. The program was split into several subprograms realizing the subsequent steps of the analytical procedure which are linked together, including PMT, EMCCD, electrochemical and stage software (as shown in Fig. S4, Supporting Information). All the programs are written in the C language and therefore occupy a minimum of the available memory space. The program runs through the list of the program steps continuously and smart software provides the fine computer interface. The diagram of the processes of the software of the ECL imaging analyzer is shown in Fig. 2.

In the PMT detection model, different working electrodes can be individually scanned and thus the current-potential curves and ECL intensity-potential curves in different channels can be obtained separately. Many types of electrochemical techniques, including potential step, linear sweep voltammetry, cyclic voltammetry, and differential pulse voltammetry, are available in the software. In the EMCCD detection model, ECL imaging at electrode array can be easily obtained at one time or individually obtained by moving the mobile stage.

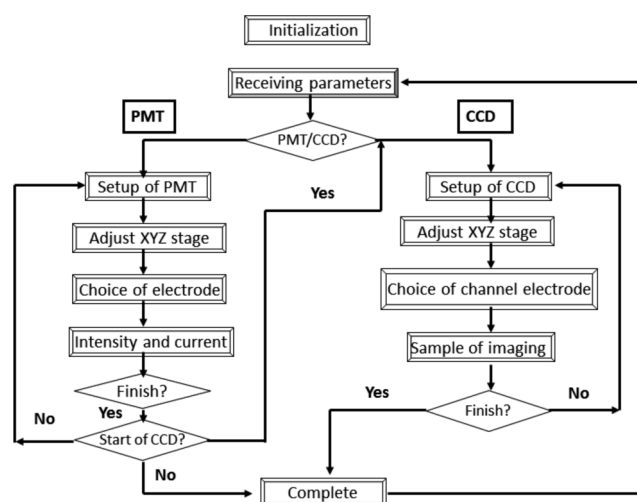


Fig. 2 Block diagram of the software of the ECL imaging analyzer.

Application of the ECL Imaging Analyzer

Electrode array

Here, we show the application of the ECL imaging analyzer to electrode array in order to verify the feasibility of the ECL imaging analyzer. As examples, two types of electrodes, including four SPCEs and also bipolar electrodes (2×3), are used in the typical ECL system, $\text{Ru}(\text{bpy})_3^{2+}$ -TPA. Cyclic voltammograms (CVs) and ECL intensity vs. potential profiles were firstly recorded in 0.1 M PBS (pH 7.4) containing

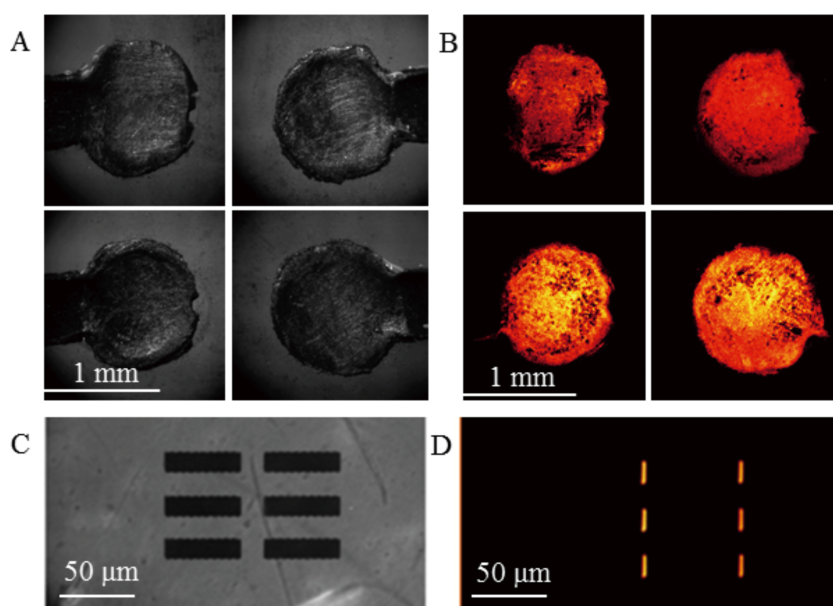


Fig. 3 The photograph in a bright field (A, C) and pseudo-color ECL imaging (B, D) in 0.1 M PBS (pH 7.4) containing 1×10^{-6} M $\text{Ru}(\text{bpy})_3^{2+}$ -50 mM TPA solution at (B) four SPCEs at an applied potential of 1.2 V to working electrodes, exposure time, 5 s; and at (D) six bipolar electrodes at an applied potential of 30 V to electrodes, exposure time, 10 s.

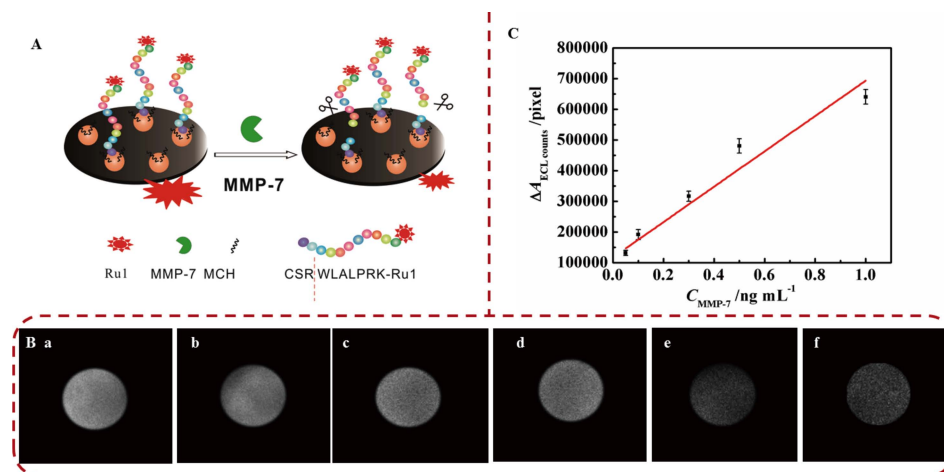


Fig. 4 (A) Scheme of the ECL bioassay of MMP-7. (B) ECL images for the detection of different concentrations of MMP-7. The concentration of MMP-7 is 0 (a), 0.05 (b), 0.1 (c), 0.3 (d), 0.5 (e), and 1 ng/mL (f). (C) Calibration curves of MMP-7.

1×10^{-6} M $\text{Ru}(\text{bpy})_3^{2+}$ and 50 mM TPA with a scan rate of 0.1 V/s at four SPCEs using a PMT model. Both of the curves of CVs and ECL can be obtained at the same time at four SPCEs (see Fig. S5 in Supporting Information). In cyclic voltammograms, four typical oxidation peaks at about +0.98 V vs. Ag/AgCl are observed, ascribed to the oxidation of TPA. Although the same electrolytes and equipment were used, the peak currents at each SPCE are slightly different. The difference of peak current is ascribed to the slightly different area of each SPCE. The four typical ECL intensity vs. potential profiles at four SPCEs are obtained, attributed to the classic oxidative-reduction coreactant mechanism.²⁷ All the obtained data using the developed ECL imaging analyzer with the PMT model are the same as that with a commercial ECL system by Xi'an Remax Electronic Co. Ltd. (Xi'an, China).

Figure 3 shows the photograph in a bright field and pseudo-color ECL imaging at four working SPCEs (A, B) and six bipolar electrodes (C, D), obtained using the ECL imaging analyzer with EMCCD detector. The photograph in a bright field can provide the information of the electrodes fabricated without an applied potential. The original ECL imaging obtained under an applied potential is grey since EMCCD (Andor iXon3) used in our instrument has a high sensitivity without color function. The average ECL counts for quantitative analysis were obtained using in-house image analysis ImageJ software. In Fig. 3(B), the calculated ECL count at each SPCE is 11088956, 11107163, 11248210 and 10731347, respectively, and thus an RSD of 3.2% at four SPCEs is obtained. This indicates that good reproducibility at four working SPCEs is feasible. The pseudo-color ECL imaging at six bipolar electrodes also is clearly observed with good reproducibility (Fig. 3(D)). The ECL light emission fields detected by EMCCD are much smaller than the area of bipolar electrodes, which is attributed to the fact that the ECL was produced only on the anodic part of the bipolar electrodes.²⁸ All results obtained support that our ECL imaging analyzer can work to get intensity-based quantitative and qualitative information at an electrode array.

Bioassay for MMP-7

To show further the quantitative analysis using our ECL imaging analyzer, an ECL biosensor for visual detection of MMP-7 was developed. The schematic diagram of the principle

of the ECL imaging detection of MMP-7 is demonstrated in Fig. 4(A). Four working SPCEs were chosen as bare electrode platform in order to shorten analysis time and improve the accuracy of the measurement. GNPs were electro-deposited onto SPCEs to facilitate self-assembly of the ECL probe Ru1-peptide onto the electrode surface. In the absence of MMP-7, a high ECL signal can be obtained at the ECL biosensor in the presence of TPA. In the presence of MMP-7, MMP-7 can specifically cleave the peptide and then lead the partly Ru1-peptide to leave the electrode surface,²⁹ resulting in the decrease of the ECL intensity obtained from the resulted electrode in the presence of TPA. Figure 4(B) shows the ECL imaging of different ECL biosensors in the presence of different concentrations of MMP-7. From Fig. 4(B), it can be seen that the ECL counts are decreased with an increase of the concentration of MMP-7. Figure 4(C) shows a linear relationship of the ECL counts with the concentration of MMP-7 in the range from 0.05 to 1 ng/mL. The linear regression equation was $\Delta A = 575971.9C + 117547.8$ (unit of C is ng/mL) with correlation coefficient of 0.9747. The results indicate that the ECL imaging analyzer could be used for the detection of biological molecules with high throughput and high sensitivity.

Conclusions

A multi-functional ECL imaging analyzer was successfully designed and constructed. The ECL imaging analyzer hardware consists of an electrochemical part, light detection part and automation movement platform part. It can be controlled by a personal computer with an in-house software program that is specifically designed for this system. The ECL imaging analyzer can effectively work for electrochemical study, ECL intensity detection at electrode array, and ECL imaging at bipolar electrodes or electrode array. The incorporation of a PMT with CCD in the multi-functional ECL imaging analyzer not only provides ECL intensity/count-based quantitative information at a single electrode or electrode array, but also the qualitative information including color and shape on electrode array with high sensitivity and short analysis time. We expect that the ECL imaging can be applied in multiplexed bioassay and imaging studies.

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Supporting Information

A schematic diagram of the preparation process of SPCEs and bipolar electrodes, a scheme of the hardware and software of the ECL imaging analyzer, and cyclic voltammograms and ECL intensity vs. potential profiles at four SPCEs. This material is available free of charge on the Web at <http://www.jsac.or.jp/analsci/>.

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