Anal Bioanal Chem
DOI 10.1007/s00216-016-9360-z

RESEARCH PAPER

Simple and sensitive electrogenerated chemiluminescence peptide-based biosensor for detection of matrix metalloproteinase 2 released from living cells

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Received: 29 October 2015 / Revised: 29 December 2015 / Accepted: 25 January 2016

Abstract A simple and sensitive electrogenerated chemiluminescence biosensor was developed to monitor matrix metalloproteinase 2 (MMP-2) by employing a specific peptide (CGPLGVRGK) as a molecular recognition substrate. Bis(2, 2′-bipyridine)-4′-methyl-4-carboxybipyridine-ruthenium *N*-succinimidyl ester-bis(hexafluorophosphate) ($Ru(bpy)_2(mcbpy\text{-}O\text{-}Su\text{-}ester)(PF_6)_2$ (Ru1) was used as ECL-emitting species and covalently labeled onto the peptide through NH_2-containing lysine on the peptide via acylation reaction to form Ru1-peptide as an ECL probe. An ECL peptide-based biosensor was fabricated by self-assembling the ECL probe onto the surface of gold electrode. MMP-2 can specifically cleave the Ru1-peptide on the electrode surface, which led the partly Ru1-peptide to leave the electrode surface and resulted in the decrease of the ECL intensity obtained from the resulted electrode in 0.1 M phosphate-buffered saline (pH 7.4) containing tri-*n*-propylamine. The decreased ECL intensity was piecewise linear to the concentration of MMP-2 in the range from 1 to 500 ng/mL. Moreover, the ECL biosensor is successfully applied to detection of MMP-2 secreted by living cell, such as HeLa cells. Additionally, the biosensor was also applied to the evaluation of matrix metalloproteinase inhibitors. The strategy presented here is promising for other disease-related matrix metalloproteinase assay and matrix metalloproteinase inhibitor profiling with sensitivity and simplicity.

Keywords Electrogenerated chemiluminescence · Biosensor · Peptide · Matrix metalloproteinase 2

Published in the topical collection *Analytical Electrochemiluminescence* with guest editors Hua Cui, Francesco Paolucci, Neso Sojic, and Guobao Xu.

Electronic supplementary material The online version of this article (doi:10.1007/s00216-016-9360-z) contains supplementary material, which is available to authorized users.

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Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteases capable of degrading extracellular matrix components and other extracellular proteins [1]. The overexpression of MMPs is reported to be closely related with tumor invasion, metastasis, and angiogenesis [2, 3]. Matrix metalloproteinase 2 (MMP-2) is one of the most vital MMPs and has been reported to be abnormal in many types of cancer, such as breast cancer [4], bladder cancer [5], and cervical cancer [6], etc. Therefore, sensitive detection of MMP-2, especially detection of MMP-2 releasing from living cell, is of great importance for clinical diagnosis and therapy of tumor in the early stage [7]. Currently, the traditional and widely used methods for the detection of MMP-2 are zymography [8, 9] and ELISAs [10, 11]. However, zymography usually offers a simple analytical tool but it is suited for qualitative rather than quantitative analysis [9]. Although ELISAs are very sensitive and can detect and quantify MMPs at low levels, ELISAs always involve tedious separation/washing processes and require the utilization of costly antibody proteins, which is not ideal for clinical diagnostics, especially in cell analysis. Therefore, the development of simple and

Published online: 03 March 2016

sensitive method for the detection of MMP-2 released from cell is needed for cancer diagnosis and therapy monitoring.

Recently, peptide has been used as a promising alternative to conventional antibody in bioassay due to its advantages, such as being reliable, cost-effective, stable, resistant to harsh environments, and more amenable to engineering at the molecular level than antibodies [12]. Several bioassays using peptide as substrate have been developed for the determination of MMP-2, such as colorimetric [13, 14], fluorescent [15–18], and electrochemical method [19–21]. These peptide-based bioassays have been used to detect MMP-2 in different clinical samples with good sensitivity and selectivity. However, challenges related to complexity and sensitivity are still present. Electrogenerated chemiluminescence (electrochemiluminescence (ECL)) is the generation of light through electrochemical process, in which involves the generation of species at electrode surfaces that then undergo electron-transfer reactions to form excited states that emit light [22]. ECL has several attractive features, including high sensitivity, simplified optical setup, good temporal and spatial control, and a very low background signal [23–29] and has been widely used in bioassay as sensitive analytical [30–33] and imaging technique [34, 35]. In spite of its excellent properties, there are no studies concerning the detection of MMPs by ECL method to our best knowledge.

The aim of the present work is to develop a simple and sensitive ECL peptide-based bioassay for the detection of matrix metalloproteinases releasing from living cell. MMP-2 was chosen as a target analyte and a MMP-2 specific peptide (CGPLGVRGK) was used as a molecular recognition substrate. Bis(2,2′-bipyridine)-4′-methyl-4-carboxybipyridine-ruthenium *N*-succinimidyl ester-bis(hexafluorophosphate) ($Ru(bpy)_2$(mcbpy-O-Su-ester)$(PF_6)_2$ (Ru1) was used as ECL emitter and covalently labeled onto the peptide via acylation reaction to form Ru1-peptide as an ECL probe. The ECL peptide-based biosensor was fabricated by self-assembling the ECL probe onto the surface of gold electrode. The schematic diagram of the principle of the ECL detection of MMP-2 is demonstrated in Fig. 1. The principle of ECL detection is on basis of the specific cleavage event of the ECL probe on the electrode surface in the presence of MMP-2 releasing from living cell. MMP-2 can specifically cleave the peptide that contains an amino acid sequence of PLGVR between G and V [36], which led the partly Ru1-peptide to leave the electrode surface and resulted in the decrease of the ECL intensity obtained from the resulted electrode in the presence of coreactant. The characteristics and the analytical performance of the ECL peptide-based biosensor were investigated. Moreover, the ECL biosensor was applied to the evaluation of matrix metalloproteinase inhibitors.

Materials and methods

Reagents and apparatus

A specific peptide CGPLGVRGK (9-mer, MW = 886.09) and ferrocene carboxylic acid (Fc)-labeled specific peptides (CGPLGVRGK -Fc, Fc-peptide) chemically synthesized were purchased from Shanghai Apeptide Co., Ltd. (China). Matrix metalloproteinase-2 (human recombinant, expressed in mouse NSO cells, 72 kDa, M9070), matrix metalloproteinase-3 human (recombinant, expressed in *Escherichia coli*, 19.5 kDa, SRP7783), matrix metalloproteinase-7 human (recombinant, expressed in *E. coli*, 28 kDa, M4565), matrix metalloproteinase-9 human (recombinant, expressed in NSO cells, 95 kDa, M8945), prostate-specific antigen (PSA; MW = 34,000, P3235), 4-aminophenylmercuric acetate (APMA; A9563), bis(2,2′-bipyridine)-4′-methyl-4-carboxybipyridine-ruthenium *N*-succinimidyl ester-bis(hexafluorophosphate) ($Ru(bpy)_2$(mcbpy-O-Su-ester)$(PF_6)_2$, abbreviated as Ru1, 96631), Brij® L23 (B4184), and dimethyl sulfoxide (DMSO; D8418) were purchased from Sigma-Aldrich (USA). Matrix metalloproteinase-10 human (recombinant, expressed in *E. coli*, ab177631) was obtained from Abcam (UK). Bovine serum albumin (BSA) was obtained from Beijing Biosynthesis Biotechnology Co., Ltd. (China). Genistein and curcumin were obtained from Aladdin (China).

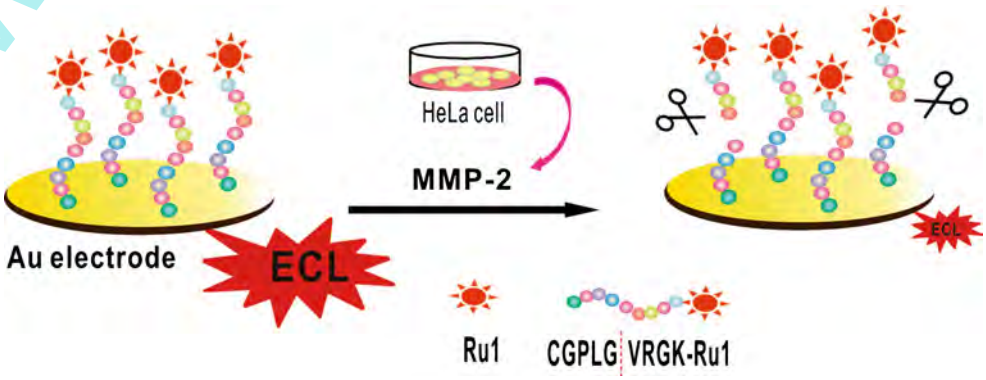

Fig. 1 The schematic diagram of the principle of the ECL detection of MMP-2

0.1 M carbonate buffer consisted of 0.1 M Na_2CO_3 and 0.1 M $NaHCO_3$(pH 8.5); 10 mM phosphate buffer (PB; pH 7.4) which contained 10 mM NaH_2PO_4 and 10 mM Na_2HPO_4 was used as the washing solution. The rest of the chemical reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Ultrapure water (Milli-Q, Millipore, 18.2 MΩ) was used to prepare all aqueous solutions.

ECL measurements were performed with a MPI-E ECL detector (Xi'an Remax Analysis Instruments Co. Ltd., China). The experimental setup for ECL measurement was the same as the previous paper [12]. A commercial cylindroid glass cell was used as an ECL cell, which contained a conventional three-electrode system consisting of either a gold electrode (Au; 2.0 mm diameter) or an ECL peptide-based biosensor 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 700 V unless otherwise stated. Electrochemical impedance spectra (EIS) were collected by CHI 660 electrochemical workstation (CH Instruments, China) and subsequently analyzed using the CHI 660 software package (CH Instruments, China). The integrated system for the ECL imaging includes a CHI 660 electrochemical workstation (CH Instruments, China) suitable to provide the needed potential for the ECL triggered reaction, an Olympus IX-51 inverted microscope (Olympus Corporation, Tokyo, Japan), and a Magnafire model iXon+ DU-897 Andor EMCCD (Andor Technology Ltd., Belfast, Northern Ireland). The ECL images were obtained under darkroom conditions, with an exposure time of 16 s. Camera control as well as image analysis were carried out using Andor SOLIS (v. 4.18, Andor Technology Ltd., Belfast, Northern Ireland). X-ray photoelectron spectroscopy (XPS) experiments were carried out on a Kratos Analytical Axis Ultra photoelectron spectrometer (Kratos Analytical Ltd., UK) with Al Kα radiation ($h\nu = 1486.68$ eV). The absorbance spectra were recorded using a 1.0-mm path length quartz cuvette on a UV–vis spectrophotometer (UV-2450, Shimadzu Corporation, Japan). Atomic force micrograph (AFM) images were obtained with a CSPM5500 Scanning Probe Microscope (Being Nano-Instruments, Ltd., China). A Bruker Maxis with an ESI source was used for mass spectrum analysis (Bruker, Germany).

Synthesis of ECL probe

The Ru1-labeled peptide (Ru1-peptide) was synthesized as the following. Briefly, 1 mg of the peptide (CGPLGVRGK, 1.1 μmol) was dissolved into 1 mL of 0.1 M carbonate buffer (pH 8.5), and 5 mg of Ru1 (4.9 μmol) was dissolved into 300 μL of DMF; 1 mL peptide, 300 μL Ru1, and 2 mL of 0.1 M carbonate buffer (pH 8.5) were mixed and stirred overnight at 4 °C. The Ru1-labeled peptides were purified by dialysis using MD36-1Da molecular (1000) weight cutoff membrane with 10 mM PB (pH 7.4) and then stored at 4 °C for use. The concentration of the Ru1-peptide solution was calculated to be 1.0×10^{-4} M according to the value of UV–vis absorption of Ru1 at 457 nm (with an extinction coefficient ε of 1.4×10^4 L mol^{-1} cm^{-1}) [37].

Fabrication of the ECL peptide-based biosensor

Prior to the experiment, a gold electrode was polished with 0.3, 0.05, and 0.01 μm alumina slurry and then ultra-sonicated in water for 5 min, and then cleaned electrochemically by a linear scanning potential between 0 and +1.5 V in 0.10 M H_2SO_4 until a stable cyclic voltammogram was obtained. After being dried with nitrogen, the cleaned gold electrode was dipped into 100 μL 10 μM Ru1-peptide solution for 3 h at room temperature and then thoroughly rinsed with 10 mM PB (pH 7.4) to remove the unbinding ECL probe. The Ru1-peptide-modified gold electrode was then immersed into 1 mM mercaptohexanol solution for 30 min. The resulting electrode was washed with water and used as the ECL peptide-based biosensor.

Determination of MMP-2

ProMMP-2 was first activated with APMA according to the protocols provided by the manufacturer (Sigma-Aldrich, USA); 100 μL ProMMP-2 (72 kDa, 10 μg) in TCNB buffer (50 mM Tris with 10 mM $CaCl_2$, 150 mM NaCl, and 0.05 %Brij 35; pH 7.5) was mixed with 10 μL 10 mM APMA. The mixture was incubated at 37 °C for 1 h with gentle shaking. The concentration of MMP-2 was calculated to be 95 μg/mL using human MMP-2 ELISA Kit (RAB0365) according to the manufacturer's instructions (Sigma-Aldrich, USA).

The ECL peptide-based biosensor was immersed into 100 μL TCNB buffer containing a certain amount of active MMP-2 for 1 h at 37 °C. The ECL measurement was performed in 1.0 mL 0.10 M phosphate-buffered saline (PBS; 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, and 0.1 M KCl, pH 7.4) contained 50 mM TPA using a triangular potential scan with the rate of 0.1 V/s. The ECL peak intensity (I) at about 0.9 V vs Ag/AgCl at initial cycle was used as ECL signal. The concentration of MMP-2 was quantified by a decreased ECL intensity ($\triangle I = I_0 - I_S$), where I_S and I_0 were the ECL peak intensity of the ECL peptide-based biosensor after and before reacted with MMP-2, respectively. In the serum sample experiment, the procedures were similar as above, except for TCNB reaction buffer containing a desired amount of serum sample.

Cell culture

HeLa cell was cultivated for 24 h in 1 mL of cell culture medium (Dulbecco's modified Eagle media (Hyclone) supplemented with 10 % (*v*/*v*) fetal bovine serum (Gibco), 1 % penicillin:streptomycin (Gibco)) at 37 °C with 5 % CO_2 in culture dish. Macrophage was cultivated for 24 h in 1 mL of cell culture medium (Dulbecco's modified Eagle media supplemented with 15 % (*v*/*v*) fetal bovine serum and 1 % streptomycin:penicillin) at 37 °C with 5 % CO_2 in culture dish. The density of cells was determined by cell counting chamber (Sino-Foreign Joint Venture Shanghai Qiu-Jing Biochemical Reagent and Instrument Co. Ltd.).

For the determination of MMP-2 in the culture media of HeLa cell, 10 μL of the culture media was collected and mixed with 90 μL TCNB buffer. Then, the ECL peptide-based biosensor was incubated with the mixture at 37 °C for 1 h. As for control, for the determination of MMP-2 in the culture media of macrophage, the ECL peptide-based biosensor was directly dipped into the culture media of macrophage. After the cleavage reaction between the ECL peptide-based biosensor and the culture media of HeLa cell and macrophages, the ECL measurement was performed as the abovementioned.

Results and discussion

Characterization of the ECL peptide-based biosensor

The schematic diagram of the principle of the ECL detection of MMP-2 is shown in Fig. 1. Ru1 was used as an ECL emitter. A MMP-2 specific peptide (CGPLGVRGK, Fig. S1 in the Electronic Supplementary Material, ESM) was employed as a molecular recognition substrate. Ru1 was covalently labeled onto the peptide through NH_2-containing lysine on the peptide via acylation reaction and was utilized as an ECL probe. In order to confirm the conjugation of Ru1 with peptide, the ECL probe synthesized was characterized by UV–vis, fluorescent, mass spectrum, and ECL techniques. UV–vis absorption spectrum of the Ru1-peptide in 100 mM PBS (pH 7.4) showed the characteristic peaks at 212, 255, 287, and 457 nm (b), corresponding to the characteristic peak of peptide at 212 nm (a), and the characteristic peaks of Ru1 at 212, 245, 286, and 457 nm (c, see Fig. S2 in the ESM). Fluorescent emission at 637 nm can be observed in Ru1-peptide solution, which is a little red shift from Ru1 (610 nm) (see Fig. S3 in the ESM). Mass spectrum of Ru1-peptide (calcd, *m*/*z* 595.5091; found, *m*/*z* 595.2712) indicates the covalent of Ru1 onto the peptide (see Fig. S4 in the ESM). ECL intensity vs potential profile of Ru1-peptide-modified gold electrode was recorded in 0.10 M PBS (pH 7.4) containing 50 mM TPA (see Fig. S5 in the ESM). Two ECL emission peaks appeared at 0.90 and 1.25 V at the Ru1-peptide-modified gold electrode in 0.1 M PBS (pH 7.4) containing 50 mM TPA when the potential is scanned from 0 to 1.4 V. This is corresponding to the classic oxidative-reduction coreactant mechanism [38]. The ECL peak at 0.90 V is mainly attributed to $Ru1^{2+}$* produced by the reaction between of TPA^+ (formed during TPA oxidation) and $Ru1^+$ (formed from the reduction of $Ru1^{2+}$ by $TPA^{\cdot}$ free radical). The ECL peak at 1.25 V is generated from the reaction between radical $TPA^{\cdot}$ and $Ru1^{3+}$ (formed from the oxidation of $Ru1^{2+}$). All of the characteristic peaks in adsorption spectrum, a red shift of fluorescence maximum wavelength, mass spectrum of Ru1-peptide, and the characteristics of ECL emission of Ru1-peptide-modified gold electrode indicate the successful labeling of Ru1 on peptide.

The ECL peptide-based biosensor was fabricated by self-assembling the ECL probe onto a gold electrode through thiol-Au bond and then blocking with MCH. The ECL peptide-based biosensor was characterized by XPS analysis for the chemical composition and status of surface, and electrochemical impedance spectroscopy for the interfacial electron-transfer behavior. As shown in Fig. 2, the presence of the C 1 s (286.0 eV), O 1 s (532.0 eV), and N 1 s (398.5 eV) peaks in the XPS data is a reliable indication that peptide is successfully modified onto the surface of gold electrode. The presence of distinct S $2p_{3/2}$ and S $2p_{1/2}$ peaks at 161.6 and 162.7 eV is commonly attributed to the self-assembly of Ru1-peptide by Au-S bond [39].

The electrochemical impedance spectra of different electrodes in the fabrication process of the ECL peptide-based biosensor were recorded. The results show that the electron-transfer resistance ($R_{et,}$ 1792 Ω) at the peptide-modified electrode is bigger than that (~108 Ω) at a bare gold electrode, indicating that the specific peptide was immobilized onto the surface of the gold electrode [40]. The R_{et} at the MCH blocked electrode decreased to 958 Ω (see Fig. S6 in the ESM). The decrease in R_{et} may be ascribed to the fact that nonspecifically adsorbed peptide is removed from the surface by MCH [41, 42]. R_{et} further decreased to 719 Ω due to the release of partly Ru1-peptide from the surface of gold electrode after reacted with MMP-2 [43]. This is consistent with the capacitance data (from 0.8, 0.45, 0.24, and 0.22 μF for bare gold electrode, peptide-modified gold electrode, MCH/peptide-modified gold electrode and MCH/peptide-modified gold electrode after reacted with MMP-2, respectively) [40, 44]. These results supported that the assembly process is successful.

Feasibility of the ECL peptide-based biosensor for MMP-2

The principle of the ECL detection of MMP-2 is based on the specific cleavage event of the ECL probe on the gold electrode surface in the presence of MMP-2 (Fig. 3a). In the absence of

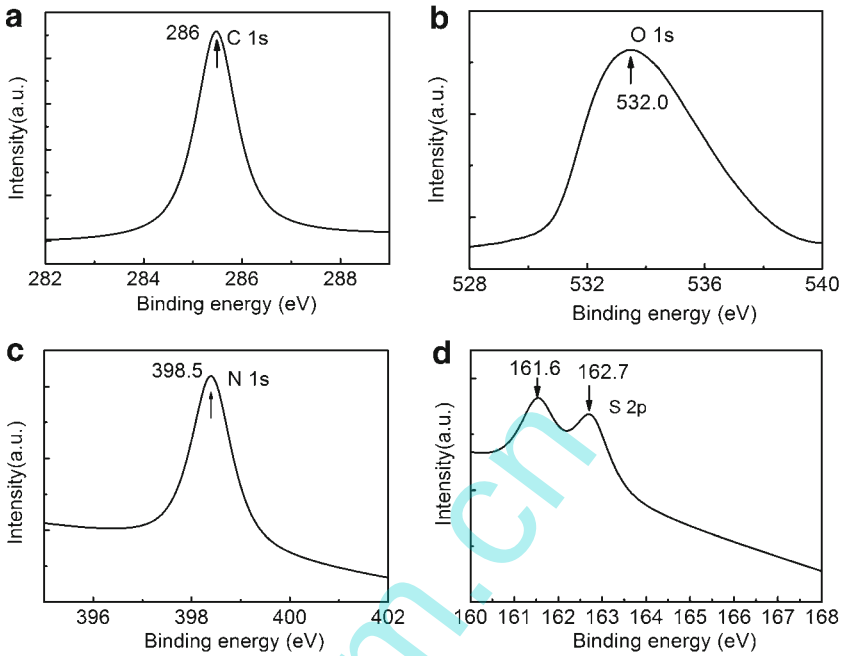

Fig. 2 High-resolution XPS spectra for C 1S region (**a**), O 1S region (**b**), N 1S region (**c**), and Au-S 2p region (**d**)

MMP-2, a strong ECL signal at 0.9 V vs Ag/AgCl was observed (10732, Fig. 3b(a)) at the ECL peptide-based biosensor in 0.1 M PBS (pH 7.4) containing 50 mM TPA. The higher ECL signal is clearly visible from the ECL imaging (Fig. 3c(a)). In presence of 50 ng/mL MMP-2, the ECL peak intensity decreases to 7246 (Fig. 3b(b)). When the MMP-2 concentration is elevated from 50 to 300 ng/mL, the ECL peak intensity further decreases to 4561 (Fig. 3b(c)). This is because MMP-2 specifically cleaved the peptide that contain an amino acid sequence of PLGVR between G and V [36], which led the partly Ru1-peptide to leave the electrode surface and resulted in the decrease of the ECL intensity obtained from the resulted electrode (Fig. 3a). This cleavage procedure can be simply visualized as ECL imagings (Fig. 3c). Further work on visualized ECL bioassay of multiple protease is going on in our lab. It should be noted that the Au-S bond is not stable at a potential as high as ca. 0.9 V, which make the ECL peptide-based biosensor use only one time [45]. The problem can be solved by covalent coupling immobilized peptide onto glassy carbon electrode [45] or by immobilizing peptide onto Nafion-AuNP-modified electrode [12]. Although there's the limit of self-assembly technique, a relative standard deviation (RSD) of 3.3 % in ECL intensity was obtained for 11 different independent ECL peptide-based biosensors (see ESM Fig. S9). Therefore, after each ECL measurement, the gold electrode was cleaned and self-assembled with fixed concentration of Ru1-peptide and MCH for fixed time.

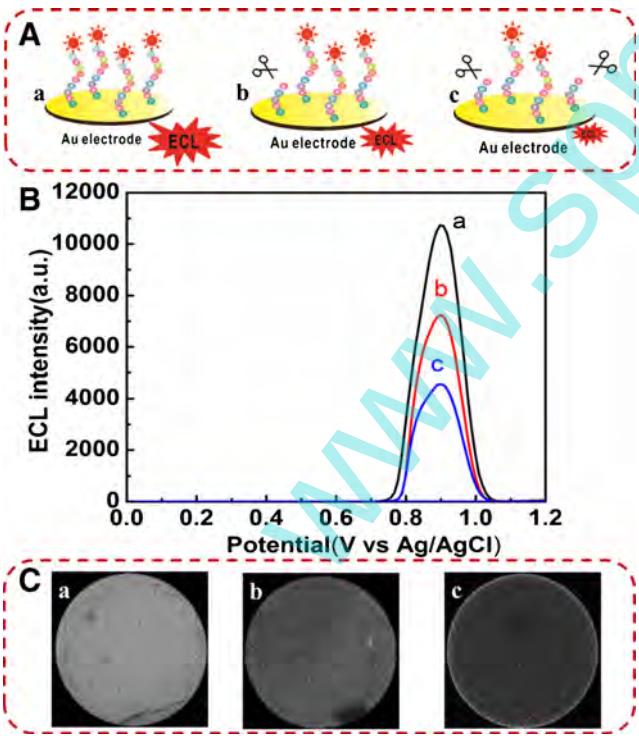

Fig. 3 (**A**) The diagram of the ECL peptide-based biosensor before and after incubation with MMP-2. (**B**) ECL intensity vs potential profiles and (**C**) ECL imaging of the ECL peptide-based biosensor before (**a**) and after incubation with 50 ng/mL MMP-2 (**b**) and 300 ng/mL MMP-2 (**c**), respectively. Cleavage time, 1 h. The measurement conditions: 0.1 M PBS contained 50 mM TPA (pH 7.4). Scan rate, 0.1 V/s

In order to confirm that the decrease of ECL intensity resulted from the cleavage of peptide by MMP-2, square wave voltammogram of Fc-peptide-modified gold electrode and AFM of Ru1-peptide-modified gold plate were recorded in the presence of MMP-2. An obvious oxidation peak at +0.4 V appears at Fc-peptide-modified electrodes in the absence and presence of MMP-2, which is attributed to the oxidation of Fc moiety on the Fc-peptide (see ESM Fig. S7). In the presence of MMP-2, the peak current decreased. AFM

image of the Ru1-peptide-modified gold plate showed that peptides were adsorbed onto the gold plate as randomly oriented monolayers with rms = 1.29 ± 0.05 nm (A). After cleavage with MMP-2, the surface was fairly smooth and the rms values decreased to 0.63 ± 0.09 nm (see ESM Fig. S8). These results indicate that the effective cleavage of peptide by MMP-2 is feasible.

To evaluate which kinds of MMP-2 are capable of specifically producing the decrease of ECL intensity of Ru1-peptide-modified electrode, the ECL peptide-based biosensor was incubated with pro MMP-2 or active MMP-2. A slight decrease in ECL intensity (6.5 %) was induced by 50 ng/mL pro MMP-2, while a strong decrease in ECL intensity was induced by 50 ng/mL active MMP-2 (32 %, Fig. S10 in the ESM), confirming that active MMP-2 does hydrolyzes peptide substrates with high specificity [36, 43].

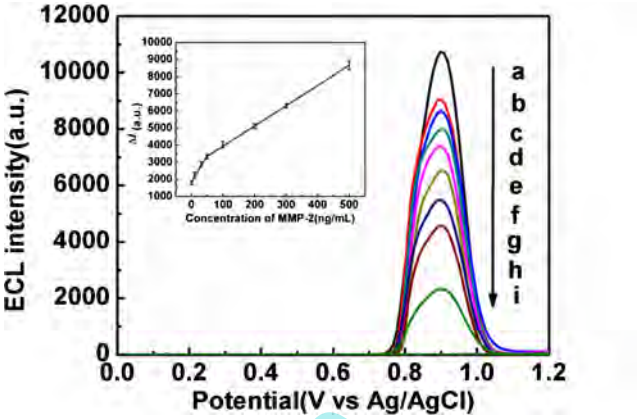

Fig. 4 ECL intensity vs potential profiles of the ECL peptide-based biosensor to different concentrations of MMP-2. (**a**) blank, (**b**) 1 ng/mL, (**c**) 10 ng/mL, (**d**) 30 ng/mL, (**e**) 50 ng/mL, (**f**) 100 ng/mL, (**g**) 200 ng/mL, (**h**) 300 ng/mL, and (**i**) 500 ng/mL. Scan rate, 0.1 V/s. Cleavage time, 60 min. *Insert* the relationship between the decreased ECL intensity and the concentration of MMP-2

Optimization condition

The dependence of the ECL intensity on the self-assembly time and cleavage time were examined. When the self-assembly time increased from 0.5 to 3 h, the ECL intensity of the ECL peptide-based biosensor increased from 1200 to 10,528. The ECL intensity reached nearly constant after the self-assembly time is up to 3 h (Fig. S11 in the ESM). Therefore, 3 h of self-assembly time was used as opmitized conditions to get high sensitivity.

Here, it was found that the ECL intensity decreased with the increase of the cleavage time. Therefore, the dependence of cleavage time, that is, the time of the incubation time of the ECL peptide-based biosensor with MMP-2, on the ECL intensity was investigated to obtain the optimum cleavage time. The results showed that the ECL intensity decreased with the increase of cleavage time from 20 to 60 min and then became stable after 60 min for 500 ng/mL (as shown in ESM Fig. S12). This suggests that 60 min was enough for 500 ng/mL MMP-2 to reach equilibrium. When the concentration of MMP-2 is decreased to 100 ng/mL, it can be seen that the cleavage time is longed to 120 min. In order to reduce the cleavage time, here, 60 min was chosen as cleavage time in the following experiments.

Analytical performance of MMP-2

In the optimized conditions, the ECL intensity decreases with the increase of concentration of MMP-2 from 1 to 500 ng/mL (Fig. 4). The decreased ECL intensity had a piecewise linear relationship with the concentration of MMP-2 in the range from 1 to 500 ng/mL. The linear regression equation was $\Delta I = 31.1C + 1837.2$ (unit of C is ng/mL) with the correlation coefficient of 0.9844 in the range of 1 to 50 ng/mL. The linear regression equation was $\Delta I = 11.8C + 2761.2$ (unit of C is ng/mL) with the correlation coefficient of 0.9996 in the range from 50 to 500 ng/mL. The detection limit is calculated to be 0.7 ng/mL MMP-2 (S/N = 3). This concentration range is of special interest for measuring the expression levels of MMP-2 in cancer research [43]. The detection limit of MMP-2 is lower than that of colorimetric [13, 14] and fluorescent [17] method (ESM Table S2). Although the detection limit is higher than the other method because of the use of nanoparticle, enzyme, or DNA-based amplification. We can lower the detection limit and improve the sensitivity by employing nanoparticles such as gold nanoparticle, carbon nanotube, or other nanomaterials [30, 33, 35, 46]. The precision was estimated for 30 ng/mL MMP-2 with five ECL biosensor and yielded reproducible results with a RSD of 3.3 %.

In this method, the cleavage time is 1 h, which is much shorter than that in ELISA (5 h or more), colorimetric (12 h) [14], fluorescence (4 h) [16], and electrochemical (16 h) [19] methods. The cleavage time can be further shortened to 20 min with the sacrifice of the detection limit. When the cleavage time is 20 min, the decreased ECL intensity has a linear relationship with the concentration of MMP-2 in the range from 50 to 800 ng/mL (ESM Fig. S13). The linear regression equation was $\Delta I = 8.7C + 2260.9$ (unit of C is ng/mL) with the correlation coefficient of 0.9929. The detection limit of MMP-2 is calculated to be 30 ng/mL.

The evaluation of the selectivity of the ECL biosensor was performed by examining the ECL response to other members of MMPs, such as MMP-3, MMP-7, MMP-9, and MMP-10, and potential interfering substances, such as BSA and PSA, respectively. ECL signal clearly demonstrated that significant decrease of ECL intensity occurred with MMP-2 while slight decreases in the ECL intensity were found for 50 times higher concentration of other tested proteins including MMP-3 (6.1 %), MMP-7 (2.9 %), MMP-9 (11.6 %), MMP-10 (5.9 %), PSA (8.9 %), and BSA (1.1 %),

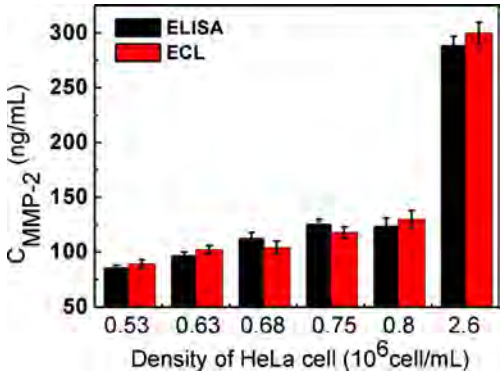

Fig. 5 Relationship between Hela cell density and the concentration of MMP-2 secreted by HeLa cells using ECL and ELISA method. The ECL measurement conditions are the same as Fig. 4. ELISA results were obtained with a commercial enzyme-linked immunosorbent assay (Sigma-Aldrich Human MMP-2 ELISA kit, RAB0365) as per the manufacturer's protocol

respectively (ESM Fig. S14). These results indicated that the ECL peptide-based biosensor was able to detect MMP-2 with a high selectivity.

Application

The potential clinical application of the ECL peptide-based biosensor in human serum samples and in the secretion of living cells was examined. Standard addition experiments were firstly performed to validate the developed method in tenfold dilution of human serum sample (ESM Table S3). Good recoveries are obtained for human serum in the range of 95.7 to 105.6 %, indicating the feasibility of the ECL method for the determination of MMP-2 in serum samples.

As is known, elevated levels of MMP-2 usually occur in the secretion of various cancer cells [47]. Here, the proposed ECL method was used to evaluate the concentration of MMP-2 secreted by HeLa cells with different densities. HeLa cells were cultured at 37 °C for 24 h in cell culture media. HeLa cells with the density of 5.3×10^5, 6.8×10^5, and 2.0×10^6 cells per milliliter could secrete 89, 104, and 300 ng/mL MMP-2, respectively, which is similar with that reported by Ma group [48]. And the concentration of MMP-2 increases with the density of HeLa cells (Fig. 5 and in the ESM Table S4, Fig. S15). Culture medium alone cannot induce the change of ECL intensity as a negative control. We directly compare the ECL results with ELISA results obtained with a commercial enzyme-linked immunosorbent assay (Sigma-Aldrich Human MMP-2 ELISA kit, RAB0365) as per the manufacturer's protocol. The standard deviations were obtained from three times measurements. The relative deviations between ECL and ELISA methods were in the range of −7.1 to 5.6 %, and the results obtained using the ECL method and ELISA method have no statistical significance when employing Student's *t* test at *P* value = 0.9, therefore indicating that the proposed ECL method is reliable for living cell testing.

As control, macrophages were cultured at 37 °C for 24 h in cell culture media and used as control living cell. There is no obvious change in ECL intensity after the ECL peptide-based biosensor incubated with macrophages secretion culture media (2~6 % decrease on the ECL intensity for 7.5×10^5 and 2.1×10^6 cells per mL) (ESM Fig. S16). This result suggests that the HeLa cells can secrete MMP-2 and macrophage cannot, and the designed ECL peptide-based biosensor is suited

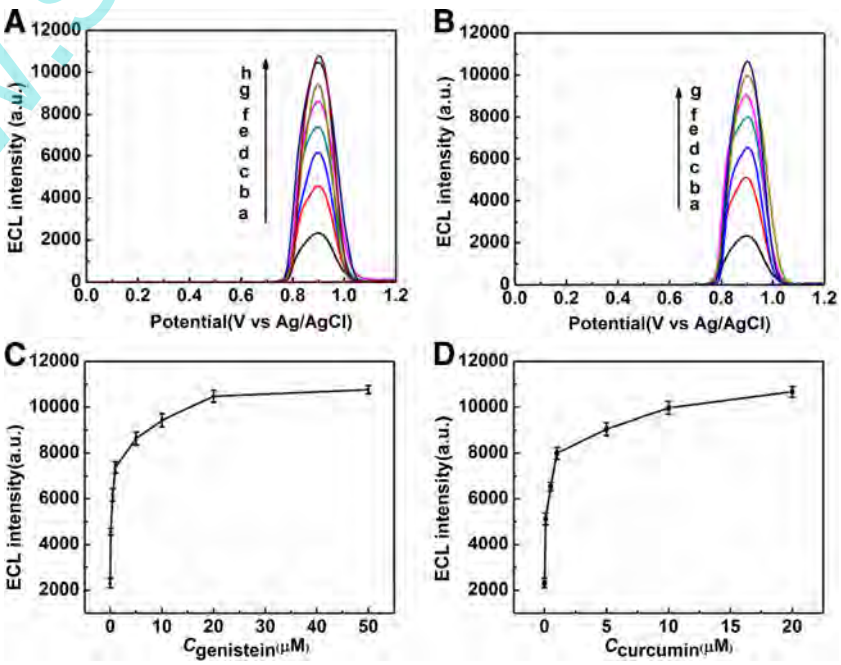

Fig. 6 (**A**) ECL intensity vs potential profiles of the ECL peptide-based biosensor after incubation with different concentrations of genistein in the presence of 500 ng/mL MMP-2. (**a**) 0, (**b**) 0.1 μM, (**c**) 0.5 μM, (**d**) 1.0 μM, (**e**) 5.0 μM, (**f**) 10 μM, (**g**) 20 μM, and (**h**) 50 μM. (**B**) ECL intensity vs potential profiles of the ECL peptide-based biosensor after incubation with different concentrations of curcumin in the presence of 500 ng/mL MMP-2 (**a**) 0, (**b**) 0.1 μM, (**c**) 0.5 μM, (**d**) 1.0 μM, (**e**) 5.0 μM, (**f**) 10 μM, and (**g**) 20 μM. The ECL intensity as function of concentrations of genistein (**C**) and curcumin (**D**). Scan rate, 0.1 V/s

for quantitative detection of MMP-2 in living cell without any complicated treatment.

To further demonstrate the potential application of the developed ECL method in the inhibition assay, the ECL peptide-based biosensor was used to quantitatively evaluate the inhibition of MMP-2 in the presence of small molecules, including genistein and curcumin at different concentrations (Fig. 6). From Fig. 6, it can be seen that an inhibitor concentration-dependent increase of ECL intensity is clearly observed and the ECL intensity increases with the increase of concentration of genistein or curcumin. The IC_{50} (inhibitor concentration producing 50 % inhibition) of genistein is estimated to be 0.91 μM. The IC_{50} of curcumin is estimated to be 0.65 μM. It indicates that the simple and sensitive ECL method is feasible in inhibition screening of MMP-2.

Conclusions

In summary, we present a simple and sensitive ECL approach for monitoring MMP-2. It has been successfully applied to the determination of MMP-2 released from living cell and screening of inhibition. The process can be finished in 1 h. A low detection limit of 0.7 ng/mL was obtained for MMP-2. The stability and sensitivity can be further improved by employing covalent immobilization method and nanomaterial amplification strategy, respectively. The strategy presented here could be easily extended to develop other ECL and electrochemical biosensing methods for other disease-related matrix metalloproteinases, and it could be employed for MMPs inhibitor profiling.

Acknowledgments We thank The National Science Foundation of China (Nos. 21522504, 21375084, 21275095, 21475082), the Natural Science Basic Research Plan in Shaanxi Province of China (No. 2014JQ2065, 2013SZS08-Z01, and 2013SZS08-P01), and the Fundamental Research Funds for the Central Universities (No. GK201505008).

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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