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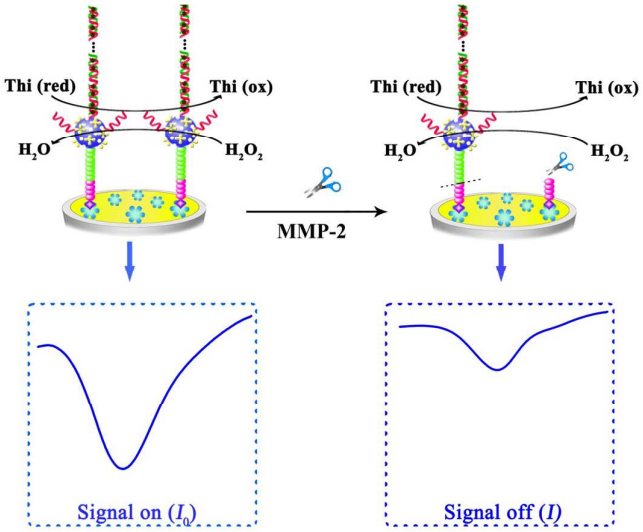

Cite this: DOI: 10.1039/c0xx00000x

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ARTICLE TYPE

A 'signal on-off' electrochemical peptide biosensor for matrix metalloproteinase 2 based on target induced cleavage of peptide

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

In this work, a 'signal on-off' electrochemical peptide biosensor was developed for the determination of matrix metalloproteinase 2 (MMP-2) on the basis of target induced cleavage of a specific peptide. The prepared single-stranded DNA-porous platinum nanoparticles-peptide (S1-pPtNPs-P1) bioconjugates were employed as nanoprobes, where the specific peptide (P1, biotin-Gly-Pro-Leu-Gly-Val-Arg-Gly-Lys-Gly-Gly-Cys) was used as a cleavage-sensing element, offering the capability of 'on-off' electrochemical signalling for the target MMP-2. As to the construction of the biosensor, S1-pPtNPs-P1 was immobilized on the electrode surface through the conjunction of biotin-streptavidin. Then, hybridization chain reaction (HCR) was triggered to embed the electroactive thionine (Thi). The pPtNPs could effectively catalyzed the decomposition of adding H_2O_2, resulting in the electrochemical signal of Thi enhanced significantly ('signal on' state). Upon sensing cleavage with MMP-2, pPtNPs and eletroactive Thi left from the electrode surface, leading to observably decreased electrochemical signal of Thi ('signal off' state). Compared with other methods detecting MMP-2, the proposed 'signal on-off' electrochemical peptide biosensor exhibited an improved sensitivity with a detection limit of 0.32 pg mL^{-1} and wide linear range from 1 pg mL^{-1} to 10 ng mL^{-1}.

Introduction

Matrix Matrix metalloproteinases (MMPs), as a therapeutically important class of zinc-dependent secreted endopeptidases, are capable of degrading various structural components of the extracellular matrix and remodeling components of surrounding tissues[1-3]. Their overexpression has been proved to be closely associated with tumor invasion, metastasis and angiogenesi[4]. As a kind of MMPs, matrix metalloproteinase 2 (MMP-2) is able to degrade type VI collagen and has been reported to be related with numerous of human tumors, including colorectal cancer, bladder cancer, and breast cancer[5,6]. Therefore, it is extremely crucial to develop rapid and sensitive methods for the quantitative detection of MMP-2 in early clinical diagnosis and therapy of tumor. Currently, common methods to detect MMP-2 are mainly involved in immunoassay[7,8] or gelatine zymography[9], in which the former is expensive and tedious, while the latter is suited for qualitative rather than quantitative analysis.

Peptides, short chains of amino acid monomers linked by peptide (amide) bonds, are simple, reliable, cost-effective and resistant to harsh environments[10-12]. More interestingly, peptide substrates containing a special amino acid sequence like Pro-Leu-Gly-Val-Arg can be specifically identified and cleaved by MMP-2 at a certain site, offering the capability to construct'signal on or off' for the target MMP-2 assays[13-15]. Unfortunately, among the detection methods, electrochemical peptide assays to detect MMP-2 have not been extensively concerned. As well known, electrochemical biosensors have been widely applied in detecting various analytes due to their merits of portable device, high sensitivity, fast response and relatively low cost[16-21]. According to the resulting increase or decrease in the electrochemical signal, most of the electrochemical biosensors have focused only on 'signal on' or 'signal off' strategy[22-25], leading to false positive electrochemical signals rooting in unneglected background signal, which affect the sensivity and selectivity of the biosensor[27,28]. The skillful combination of the 'on-off' tactics endows the biosensor with preferable abilities, including high sensitivity, good selectivity and low background signal.

Herein, a 'signal on-off' electrochemical peptide biosensor for MMP-2 assay was proposed based on target induced cleavage of a specific peptide. The peptide designed was labeled with biotin and consisted of a special amino acid sequence (biotin-Gly-Pro-Leu-Gly-Val-Arg-Gly-Lys-Gly-Gly-Cys, P1), containing a specific site which can be cleaved by MMP-2. Through the strong interaction between porous platinum nanoparticles (pPtNPs) with $-NH_2$ in S1 and -SH[29,30] of terminal Cys residue in P1, S1 and P1 were immobilized onto the surface of pPtNPs, resulting in the formation of the bioconjugates (S1-pPtNPs-P1). Through the specific conjunction of biotin-streptavidin[31,32], S1 and biotin-labeled P1 modified pPtNPs nanoprobes (S1-pPtNPs-P1) were spotted on the electrode surface. In the presence of ssDNA

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strands of S1 and S2, hybridization chain reaction (HCR) could be triggered with the generation of dsDNA polymers. Then, a large amount of positive charged electroactive of thionine (Thi) was intercalated into dsDNA grooves with negative charge to generate the electrochemical signal. With the addition of H_2O_2, pPtNPs could effectively catalyze the decomposition of H_2O_2, which significantly promoted the electron transportation of Thi ('signal on' state). When target MMP-2 was incubated onto the electrode surface, it can specifically recognize and cleave the peptide labeled with pPtNPs at a certain site between Gly and Val [13-15], resulting in the release of Thi and pPtNPs from the electrode surface and the dramatic decline of electrochemical signal of Thi ('signal off' state). Through monitoring current response signals of the electrochemical peptide biosensors incubated with different concentrations of MMP-2, the target could be quantitatively determined. The proposed electrochemical biosensor coupled 'signal on', 'signal off', HCR and pPtNPs catalysis amplified strategies achieved high sensitivity, satisfactory stability and selectivity, providing a new model for proteases detection in the peptide biosensing system.

Experimental

Reagents and materialsals

Chloroauric acid ($HAuCl_4$), chloroplatinic acid (H_2PtCl_6), thrombin (TB), bovine serum albumin (BSA), L-cysteine (L-cys), thionine (Thi), human IgG were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Ascorbic acid (AA), sodium borohydride ($NaBH_4$) and glucose were purchased from Chemical Reagent Co. (Chongqing, China). Streptavidin (SA) was bought from Shanghai Hualan Chemical Technology Co. Ltd. (Shanghai, China). Matrix metalloproteinase-2 (MMP-2) was obtained from Sino Biological Inc. (Beijing, China). The MMP-2 specific peptide and the DNA oligonucleotides (S1 and S2) were synthesized by Sangon Biotech. Co. Ltd. (Shanghai, China) with the following sequences:

Peptide (P1): Biotin-Gly-Pro-Leu-Gly-Val-Arg-Gly-Lys-Gly-Gly-Cys

S1: 5'-NH_2-TGA CAT TTG CTC GAT TCC TAT ACG AGT GGC TAT CTT TCG TCT AAT TGG CAC GAT ATG TCG-3'

S2: 5'-ACG AAA GAT AGC CAC TCG TAT TCA TCA CTG GAC CGA TAC GCG ACA TAT CGT GCC AAT TAG-3'

Other chemicals employed were all of analytical grade and directly used as received. Tris-HCl buffer (20 mM, pH 7.4) consists of 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl and 140 mM NaCl, serving as DNA oligonucleotides buffer. Phosphate buffered solution (PBS) with different pH serving as working buffer throughout the experiment was prepared with 0.1 M KH_2PO_4, 0.1 M Na_2HPO_4 and 0.1 M KCl. TCNB buffer (50 mM Tris with 10 mM $CaCl_2$, 150 mM NaCl and 0.05% Brij 35; pH 7.5) was employed in the experiment.

Apparatus

Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were carried out in a CHI-1040C electrochemical workstation (Shanghai Chenhua Instrument, China) with a conventional three-electrode system using the modified glassy carbon electrode (GCE, Φ = 4 mm) as working electrode, a platinum wire as auxiliary electrode and a saturated calomel electrode (SCE) as reference electrode. The pH of tested solution was measured with a pH meter (MP 230, Mettler-Toledo, Switzerland). Scanning electron microscopy (SEM) images were obtained from a scanning electron microscope (SEM, S-4800, Hitachi, Tokyo, Japan). Transmission electron microscopy (TEM) images were taken with a JEM 1200EX electron microscope under 120 kV accelerating voltage (H600, Hitachi, Japan). X-ray photoelectron spectroscopy (XPS) analysis was taken with the VG Scientific ESCALAB 250 spectrometer using Al Ka X-ray (1486.6 eV) as the light source.

Polyacrylamide gel electrophoresis analysis of HCR

HCR between ssDNA S1 and S2 was investigated by using polyacrylamide gel electrophoresis. Before that, S1, S2 and dsDNA formed by HCR of S1 and S2 were all heated to 95 °C for 2 min and slowly cooled to room temperature. Then, gel electrophoresis analysis was performed in the freshly prepared non-denaturing polyacrylamide gel (16%) at 100 V for 60 min in 1×TBE buffer (pH 8.3). After staining by ethidium bromide, the gel was transferred into a dark box and the electrophoresis image was obtained by a digital camera under the UV light illumination.

Synthesis of porous platinum nanoparticle

Porous platinum nanoparticles (pPtNPs) were synthesized by a gentle seed-free method[33]: 50 μL NaOH (1.0 M) solution and 3 mL fresh ascorbic acid (10 mM) aqueous solution were seriatim added to 2 mL H_2O, followed by 4 mL H_2PtCl_6 (2 mM). The mixture was stirred for about 1 min and then kept in 60 °C for 1 h. After centrifugation and washed several times with double distilled water and ethanol, the prepared pPtNPs were redispersed in 2 mL double distilled water.

In order to compare the different catalysis capacity, non-porous Pt nanoparticles (PtNPs) were prepared as following. 400 μL H_2PtCl_6 (2 mM) was diluted into 2 mL H_2O, followed by dropwise addition of 1 mL freshly prepared $NaBH_4$ (0.1 M). Then the resulting mixture was vigorously stirred for 1 h. Finally, PtNPs were collected by centrifugation and washing for three times with double distilled water.

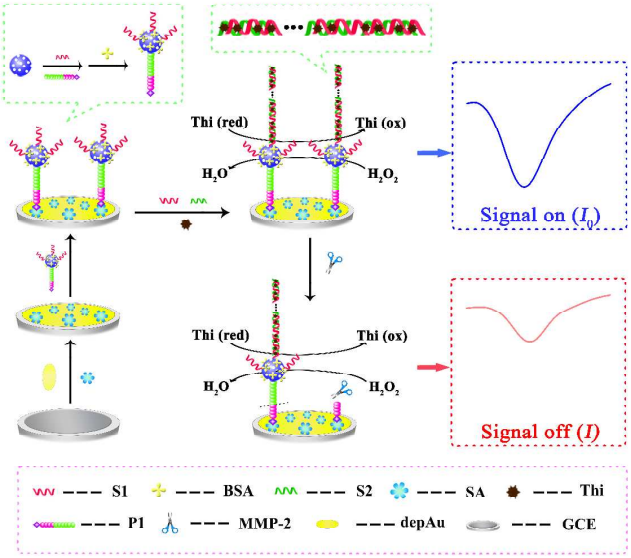
Synthesis of S1-pPtNPs-P1 nanoprobes

50 μL P1 (25 μM) and 200 μL S1 (2.5 μM) were added into 1 mL pPtNPs solution and gently stirred for 12 h at 4 °C. After that, P1 and S1 were bound to pPtNPs surface through Pt-thiol and Pt-amino interaction. Then, 50 μL BSA (1%) was added into the resultant solution and kept for 40 min to block the remaining active sites of pPtNPs. The unconjugated P1, S1 and BSA were removed by further centrifugation and washing for several times, and the obtained S1-pPtNPs-P1 nanoprobes were resuspended in 1.0 mL PBS (pH 7.0) and stored at 4 °C for further use.

Meanwhile, to investigate the superior performance of the proposed nanomaterial-amplified 'signal on-off' peptide biosensor, other two nanoprobes Thi-pPtNPs-P1 and S1-PtNPs-P1 were also prepared using the similar method. The former was obtained by conjugating pPtNPs with P1 and Thi in the absence of S1; the latter was prepared by labelling PtNPs with S1 and P1.

Fabrication of proposed electrochemical peptide biosensor

Prior to the experiment, a GCE was polished with 0.3 and 0.05 μm alumina slurry, ultrasonicated in double distilled water and ethanol, and dried in air. The cleaned GCE was electrodeposited in 2 mL $HAuCl_4$ (1%) aqueous solution under –0.2 V for 30 s (depAu/GCE). Next, in order to immobilize S1-pPtNPs-P1 nanoprobes onto the resulting electrode surface through the specific interaction between SA and biotin labelled in P1, 10 μL SA (2.0 mg mL^{-1}) was incubated for 12 h, followed by 20 μL S1-pPtNPs-P1 for 40 min at ambient temperature. Next, the introduction of 20 μL mixture containing 2.5 μM S1, 2.5 μM S2 and 1.0 mM Thi triggered HCR in situ, resulting in the formation of dsDNA and the embedding of Thi into dsDNA. After 2 h, the modified electrode was rinsed thoroughly with double distilled water. Finally, 20 μL MMP-2 with different concentrations was incubated for 2 h at 37 °C. After rinsed with TCNB buffer, the proposed peptide biosensor was prepared and ready for measurement. The stepwise fabrication process and the detection mechanism are shown in Scheme 1.



Scheme 1. Preparation process of the 'signal on-off' electrochemical peptide biosensor for MMP-2 and the detection principle. I_0 and I were DPV peak current of the proposed biosensor before and after incubation with MMP-2.

Electrochemical measurement

The modification process of the proposed biosensor was characterized by CV measurement from −0.2 V to 0.6 V in 5.0 mM $(Fe(CN)_6)^{3-/4-}$ (pH 7.4) in the absence of electroactive Thi, and from −0.5 V to 0.1 V in 0.1 M PBS (pH 7.0) in the presence of Thi. The optimization of experimental condition and the electrochemical characteristics were analyzed by DPV measurement from −0.5 V to 0.1 V with 50 mV amplitude and 0.05 s pulse width in 0.1 M PBS (pH 7.0) with or without 2.22 mM H_2O_2. The effect of MMP-2 concentrations on the electrochemical response was investigated by a decreased DPV signal ($\Delta I = I - I_0$), where I_0 and I were DPV peak current of the proposed biosensor before and after incubated with different concentrations of MMP-2. All experiments were carried out at room temperature.

Results and discussion

Characterizations of prepared nanoparticles

The SEM and TEM images of pPtNPs are shown in Fig. 1A and C, respectively. Obviously, pPtNPs were of spherical nanostructure with rough and porous surface. Based on the TEM images in Fig. S1A, the size distributing histogram of the prepared pPtNPs was counted and presented in Fig. S1B. As can be see, the particle size distribution was similar to normal distribution, and the majority size of pPtNPs was ranged from 54.4 nm to 67.2 nm. Moreover, according to the different surface morphology before and after combination with protein or other biomolecules, SEM characterization could be employed to prove the successful modification of biomolecules on the surface of materials[34-38]. Fig. 1B showed the SEM images of the nanoprobes P1-pPtNPs-S1 obtained by immobilizing S1 and P1 onto the surface of pPtNPs. A blurry surface could be clearly observed, which might be attributed to the effective attachment of S1 and P1 on the pPtNPs surface.

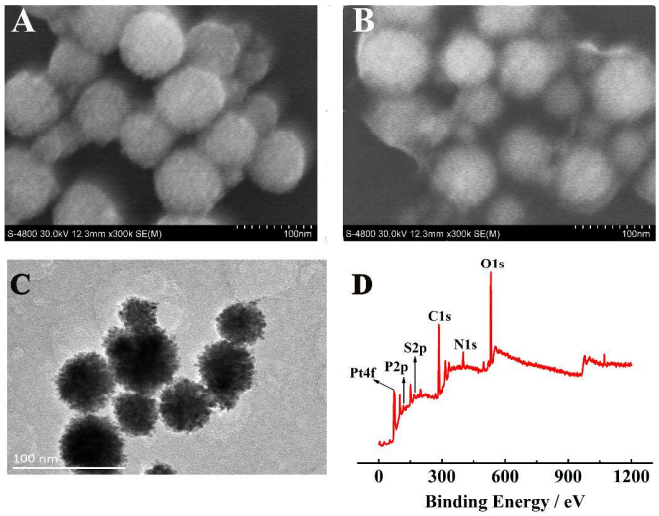


Fig. 1 SEM of the prepared pPtNPs (A) and P1-pPtNPs-S1 (B). TEM of the prepared pPtNPs (C). XPS spectra of pPtNPs immobilized with S1 and P1 (D).

The immobilization of S1 and P1 onto the pPtNPs surface was further proved by measuring X-ray photoelectron spectroscopy (XPS) of pPtNPs after loaded with S1 and P1. And the results are shown in Fig. 1D and Fig. S2 (B, C and D). The characteristic peaks of Pt4f were located at 71.5 and 74.7 eV (see ESI†, Fig. S2A), indicating the successful preparation of pPtNPs. The bands

at 401.3, 285.1 and 532.4 eV were attributed to binding energy of N1s, C1s and O 1s, respectively (Fig. S2B, C, D). The core level spectrum of S2p at 168.5 eV (Fig. S2E) was mainly obtained from P1. Because of the existence of P in S1, the peak of P2p was observed at 133.3 eV (Fig. S2F). All the elemental results confirmed that S1 and P1 have been successfully assembled onto the surface of pPtNPs.

Electrochemical characterization of the aptasensor

The electrochemical characteristics of the proposed biosensor were investigated by cyclic voltammetry (CV) and the results are shown in Fig. 2. CVs of the first three modified processes tested in 5 mM $[Fe(CN)_6]^{3-/4-}$ solution (Fig. 2A) indicated the redox current of GCE electrodeposited with AuNPs (curve b) exhibited increment compared with bare GCE (curve a), suggesting the improvement of AuNPs to the electron transfer. Next, SA assembled to the resultant electrode surface caused obvious decrease of response current (curve c), because SA with non-electroactive character greatly hindered electron transfer. Meanwhile, CV response of rest modified processes was carried out in 0.1 M PBS. From Fig. 3B, after the S1-pPtNPs-P1 nanoprobes were incubated, no redox peak was observed (curve d), due to the lack of electroactive media on the electrode surface. When a mixture of S1, S2 and Thi was further incubated, an increased redox response current could be clearly noticed (curve e), owing to the successful embedding of Thi into dsDNA formed by HCR between S1 and S2. Subsequently, the appearance of target MMP-2 (0.1 ng mL^{-1}) induced a distinct decrease of electrochemical signal (curve f), which may originate from the leaching of Thi from the electrode surface. That's because MMP-2 specifically identified and cleaved P1 at a certain site. Moreover, the HCR of S1 and S2 was verified by gel electrophoresis (see ESI†).

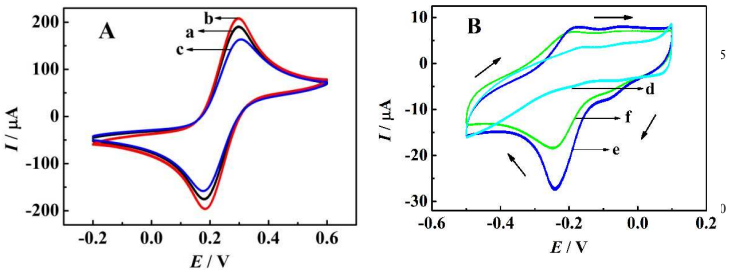


Fig. 2 (A) CVs in 5.0 mM $[Fe(CN)_6]^{3-/4-}$ (pH 7.4) of bare GCE (a), depAu/GCE (b), SA/depAu/GCE (c). (B) CVs in 0.1 M PBS (pH 7.0) of S1-pPtNPs-P1/SA/depAu/GCE (d), the resulting electrode treated with the mixture of S1, S2 and Thi (e) and followed by MMP-2 (f). Scan rate is 100 mV s^{-1}.

The superior performance of the proposed nanoprobes

To demonstrate superior performance of the proposed nanoprobes, the peptide biosensor employing S1-pPtNPs-P1 as nanoprobes was compared with those of other two nanoprobes: Thi-pPtNPs-P1 and S1-PtNPs-P1. DPV responses were measured in 0.1 M PBS (pH 7.0) with 2.22 mM H_2O_2 before and after incubation with 0.1 ng mL^{-1} MMP-2. As shown in Fig. 3A, the DPV currents of 'signal on' state (I_0) and 'signal off' state (I) were relatively lower and the peak current changed only about 6.35 μA for Thi-pPtNPs-P1. This could be ascribed to the absence of S1 in this nanoprobe, and HCR between S1 and S2 could not be triggered. Therefore, the electrochemical response could not be effectively amplified in spite of the great catalysis of pPtNPs to the reduction of H_2O_2. In Fig. 3B, higher value of I_0 and I were obtained. The DPV peak current changes about 10.42 μA, which suggested that the trigger of HCR could bring a larger change of the electrochemical response due to the presence of S1 in S1-PtNPs-P1. However, the introduction of the proposed S1-pPtNPs-P1 caused the highest I, I_0 and much more specific variety of DPV response which was 21.08 μA (Fig. 3C). This superior performance of the proposed nanoprobes may be mainly originated from two points: Firstly, owing to the presence of S1 in S1-pPtNPs-P1, HCR between S1 and S2 was triggered by the formation of dsDNA with negative charge groves, in which huge amounts of electroactive Thi with positive charge was embedded through electrostatic attraction. Secondly, compared with non-porous PtNPs, pPtNPs with much more surface area are more capable of catalyzing the decomposition of H_2O_2 and could immobilize more S1, and induce the embedding of more plentiful Thi into the formed dsDNA. Therefore, the enhanced response signal and improved sensitivity could be successfully achieved for the proposed biosensor, benefited from HCR and pPtNPs.

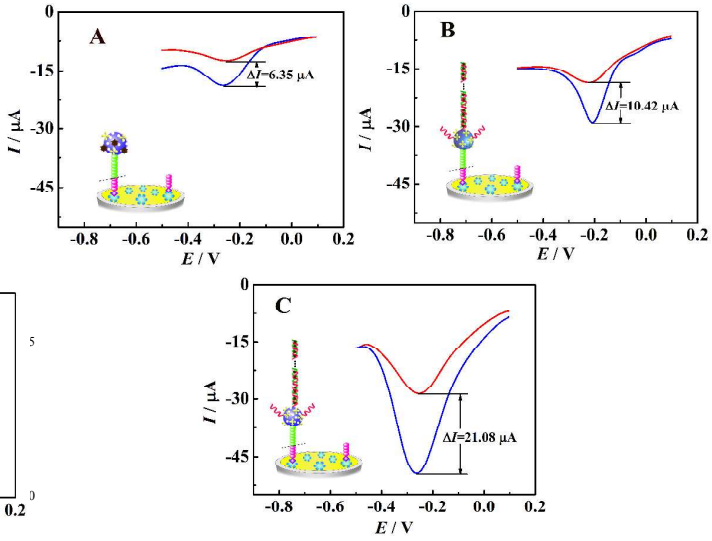


Fig. 3 DPV responses of the biosensors prepared by using different nanoprobes: Thi-pPtNPs-P1 (A), S1-PtNPs-P1 (B), S1-pPtNPs-P1 (C) in 0.1 M PBS (pH 7.0) before (blue line, I_0) and after (green line, I) incubation with 0.1 ng mL^{-1} MMP-2 in the presence of 2.22 mM H_2O_2.

Determination of MMP-2 by DPV

Under the optimal experiment conditions (see ESI†), the proposed biosensor was incubated with different concentrations of MMP-2. Then the DPV was measured in 0.1 M PBS (pH 7.0) containing 2.22 mM H_2O_2. As shown in Fig. 4A, with increasing of MMP-2 concentrations from 0 to 10 ng mL^{-1}, DPV response signal (I_0 or I) gradually decreased. And the result in Fig. 4B indicated the difference of DPV peak current ($\Delta I = I - I_0$) was

proportional to the logarithm of the MMP-2 concentration in the range of 1 pg mL^{-1} to 10 ng mL^{-1} . The linear equation was ΔI (μA) = 8.048 lg$c$ (pg mL^{-1}) + 4.866 with a correlation coefficient of 0.995. The estimated detection limit for the proposed biosensor was calculated as 0.32 pg mL^{-1} (LOD = 3 s_B/m, where s_B is the standard deviation of the blank and m is the slope of the corresponding calibration curve). Compared with other MMP-2 biosensors developed by such reported methodologies as fluorescence, photoluminescence etc, the result summarized in Table S1 (see ESI†) indicated that the proposed 'signal on-off' electrochemical peptide biosensor for MMP-2 assay showed wider dynamic linear range, lower detection limit and higher sensitivity.

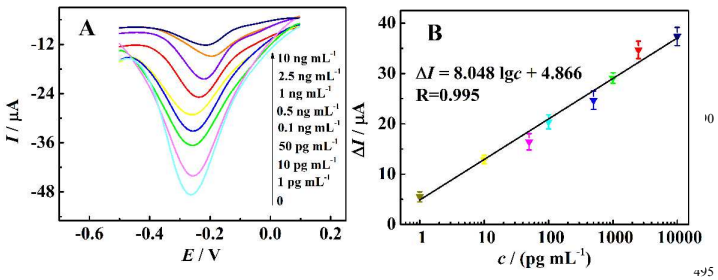


Fig. 4 (A) DPV responses of the proposed biosensor for different concentrations of MMP-2 in 0.1 M PBS (pH 7.0) with 2.22 mM H_2O_2. (B) The resultant linear calibration curve for MMP-2 concentration. Error bars: SD, n = 3.

Specificity, stability and reproducibility of the electrochemical peptide biosensor

The selectivity of the proposed biosensor was investigated by testing electrochemical response of MMP-2 (0.1 ng mL^{-1}) against other interferents, including TB (10 ng mL^{-1}) and $MgCl_2$, KCl, IgG, glucose, L-cys and BSA with the same concentration of 10 mg mL^{-1}. Fig. 5 displays ΔI of DPV response of the proposed biosensors before and after incubated with MMP-2 and other interferents, respectively. Obviously, the result obtained by three parallel measurements (n = 3) showed negligible cross-reactivity of the developed biosensor to the tested interferents even at a concentration many times higher than that of MMP-2, indicating the proposed peptide biosensor possessed much higher specificity for MMP-2.

After the proposed biosensor was incubated with 0.1 ng mL^{-1} MMP-2, the continuously CV scan of 50 cycles was measured in 0.1 M PBS (pH 7.0) with the potential range from −0.5 V to 0.1 V. As shown in Fig. S5, a 6.98% decrease of the initial response current was observed, indicating a satisfactory stability. In addition, DPV responses were recorded for six electrodes prepared under the same conditions and incubated with 100 pg mL^{-1} MMP-2. The electrochemical signals of all the electrodes only showed a relative standard deviation (RSD) of 6.3%, demonstrating an acceptable reproducibility.

Analytical application of the biosensor

The practical applicability of the proposed biosensor was investigated by using the standard addition method to detect MMP-2 in real serum sample. Different concentrations of MMP-2 solution were separately added into 10-fold-diluted serum sample (the Xinqiao Hospital, the Third Military Medical University, Chongqing, China), and DPV responses were measured. As shown in Table 1, the recovery ranged from 92.3% to 107% with RSD in the range of 3.5%-6.9%, which proved a great potential of the proposed electrochemical biosensing platform to detect MMP-2 in real samples.

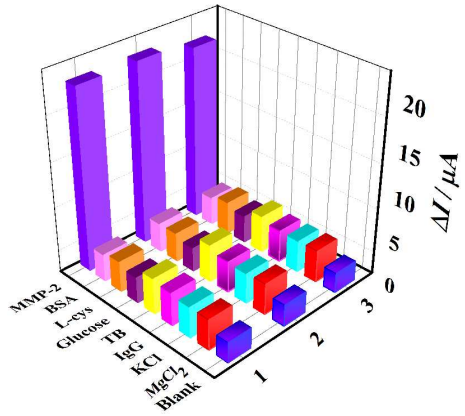


Fig. 5 The DPV responses of three batches of the proposed biosensor for MMP-2 (0.1 ng mL^{-1}) against TB (10 ng mL^{-1}) and $MgCl_2$, KCl, IgG, glucose, L-cys and BSA with the same concentration of 10 mg mL^{-1} in 0.1 M PBS (pH 7.0) with 2.22 mM H_2O_2.

Table 1 Determination of MMP-2 added in human blood serum with the proposed biosensor[a] (n = 3).

Samples	Added MMP-2	Found MMP-2	Recovery (%)	RSD (%)
1	10.0 pg mL^{-1}	10.3 pg mL^{-1}	103	4.8
2	50.0 pg mL^{-1}	46.7 pg mL^{-1}	93.4	6.9
3	0.10 ng mL^{-1}	0.92 ng mL^{-1}	92.0	3.5
4	0.50 ng mL^{-1}	0.53 ng mL^{-1}	107	5.8
5	1.00 ng mL^{-1}	1.06 ng mL^{-1}	106	4.2

[a] DPV measurements were carried out in 0.1 M PBS (pH 7.0) containing 2.22 mM H_2O_2 in the range of −0.5 to 0.1 V.

Conclusion

In conclusion, a 'signal on-off' electrochemical peptide biosensor for MMP-2 assay was successfully fabricated based on target induced cleavage of peptide. The DPV response of the prepared electrode changed before and after incubation with MMP-2 determined the 'on' and 'off' states of this biosensor. Direct conversion of a signal related to peptide cleavage into an electrochemical signal provided simple, easy and sensitive method for the determination of MMP-2. With skilful integration

of 'signal on', 'signal off', HCR and pPtNPs catalysis amplified strategies, the proposed assay platform showed higher sensitivity, more favourable specificity and stability for MMP-2 compared with other methodologies. Therefore, the proposed protocol exhibited great application prospects not only for MMP-2 also for other MMPs or proteases by simply switching substrate peptide probes.

Acknowledgements

This work was financial supported by NNSF of China (21275119) and Fundamental Research Funds for the Central Universities (XDJK2013A008).

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