Electrochemistry Communications 118 (2020) 106808

Contents lists available at ScienceDirect

Electrochemistry Communications

journal homepage: www.elsevier.com/locate/elecom

A highly selective electrochemical assay based on the Sakaguchi reaction for the detection of protein arginine methylation state

Mengyuan He[a], Jiarong Guo[a], Jiahua Yang[a], Yang Yang[a], Songyan Zhao[a], Qiao Xu[b], Tiangxiang Wei[b], Davide Maria Ferraris[c], Tao Gao[a,*], Zhigang Guo[a,*]

[a] Jiangsu Key Laboratory for Molecular and Medical Biotechnology, College of Life Sciences, Nanjing Normal University, Nanjing 210023, PR China
[b] School of Environment, Nanjing Normal University, Nanjing 210023, PR China
[c] Dipartimento di Scienze del Farmaco, Università del Piemonte Orientale, Largo Donegani 2, 28100 Novara, Italy

ARTICLE INFO

Keywords:
Electrochemical assay
Protein arginine methylation
Sakaguchi reaction
Biosensor

ABSTRACT

Protein arginine methylation is a common form of post-translational modification that plays an important role in many bioprocesses. However, research advances in this field have been severely hampered by the lack of a quick and sensitive method for detecting the arginine methylation state of a protein. In this work we propose a direct and sensitive electrochemical method for identifying the arginine methylation state. This novel assay combines an electrochemical technique with the Sakaguchi reaction, which is highly selective towards the arginine methylation state. We show that the presence of a methyl group on the arginine residue of a protein prevents the Sakaguchi reaction, while the unmethylated arginine residue selectively reacts with 8-hydroxyquinoline; the electrical signal of the reaction product is used for electrochemical detection. From this, a highly selective and simple electrochemical sensor has been developed based on (1) the high selectivity of the Sakaguchi reaction towards the arginine methylation state, and (2) the sensitive electrochemical signal generated by the linked 8-hydroxyquinoline. The assay described in this work thus provides a convenient tool for detection of protein arginine methylation, which may facilitate studies of the biological functions of protein arginine methylases and demethylases.

1. Introduction

Protein arginine methylation, a common post-translational modification (PTM) catalyzed by protein arginine methyltransferases (PRMTs) [1,2], has attracted interest in recent years because of its critical regulatory functions in pre-mRNA splicing, mRNA translation, cell signaling, DNA damage response, and cell fate decision [3]. As the existence of methylarginine demethylases (RDMs) is still under debate, the identification of the methylation state of protein arginine requires extensive investigation. Determination of the arginine methylation state of a protein is therefore essential in biomedical studies to advance our understanding of protein arginine methyltransferases (the epigenetic "writers"), arginine demethylases (the "erasers"), and the interacting proteins (the "readers") [4–6].

The detection of methylated arginine is hampered by technical difficulties due to the intrinsic physicochemical properties of the methyl group on arginine residues. From a molecular perspective, when an arginine residue is modified by addition of a methyl group [2], it causes an increase in bulkiness and hydrophobicity, which hampers the formation of hydrogen bonds [7], and thus changes protein-protein interactions [8]. However, this modification differs from other PTMs such as phosphorylation or acetylation, in that the charge is not altered after modification [9]. Moreover, the physicochemical alterations caused by methylation of the arginine guanidine group are difficult to detect and to distinguish from lysine and histidine methylation.

Current methods used for the pre-isolation of arginine-methylated proteins based on ion-exchange chromatography are largely ineffective [10]. The most effective techniques for the detection of methylated arginine rely on labeling with a heavy-methyl stable isotope [11], specific antibodies [12-14], mass spectrometry [8,15,16], PRMT inhibitors, or radioactively labeled *S*-adenosyl-L-[methyl-3H]-methionine ([3H]-AdoMet) [17,18]. However, the selectivity of these assay methods is unsatisfactory, and they rely on indirect, costly, and labor-consuming procedures. To this end, a direct and specific method for detecting protein arginine methylation would be of great utility.

Analytical electrochemical methods are usually fast, convenient, and sensitive [19] and have already been used for detecting protein PTMs [20–24] and protein arginine deiminase 4 (PAD4) [25]. We

* Corresponding authors.
E-mail addresses: taogao@njnu.edu.cn (T. Gao), guozgang@gmail.com (Z. Guo).

https://doi.org/10.1016/j.elecom.2020.106808
Received 18 July 2020; Received in revised form 25 July 2020; Accepted 25 July 2020
Available online 29 July 2020
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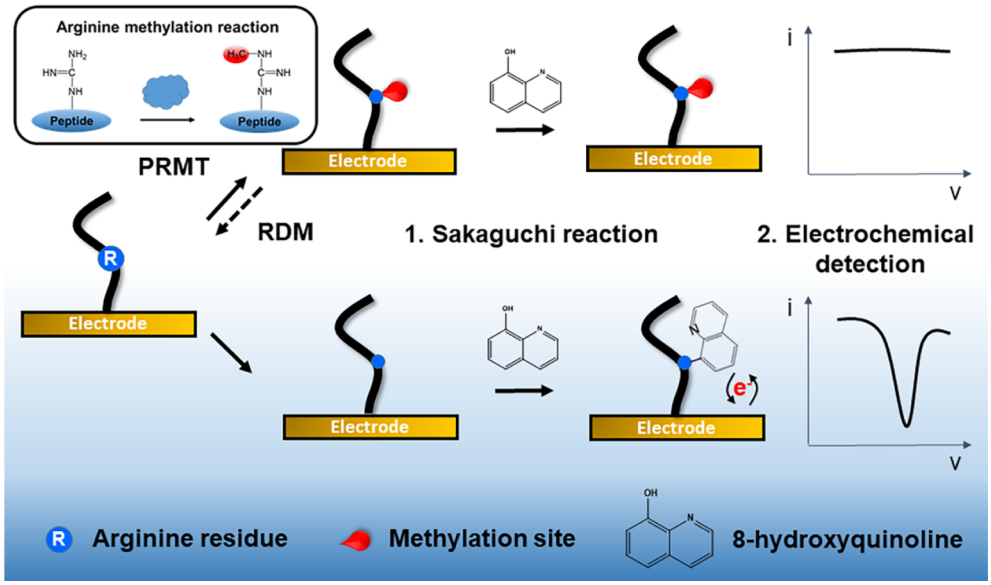

Scheme 1. Schematic illustration of an electrochemical method for the assay of the protein arginine methylation state.

propose herein a highly selective and convenient electrochemical method for detecting the methylation state of arginine. This assay method is based on our observation that the methylation state of the arginine can be distinguished by the Sakaguchi reaction. Specifically, the methylation of arginine can block this reaction, while the unmethylated guanidine group of arginine can react with 8-hydroxyquinoline (8-HQ) with extremely high selectivity through the improved Sakaguchi reaction [26], which is superior to the α-naphthol-based Sakaguchi reaction. The 8-HQ selective reaction with unmethylated arginine forms an electroactive compound that behaves as an oxidation–reduction indicator [27], making electrochemical detection possible.

Scheme 1 shows the principle of the assay. A substrate peptide that contains the arginine residue is bound to an electrode surface and is subjected to the Sakaguchi reaction. A protein with methylated arginine is not capable of performing the Sakaguchi reaction with 8-HQ and hence does not produce electrical signals. Consequently, the methylation state of the protein arginine can be traced through electrochemical measurements. This assay method is simple, does not require complex labeling or synthesis of reporting molecules, and can be used for the detection of methyltransferase activities (such as PRMT) and for the screening of enzyme inhibitors. Similarly, as the existence of N^{ω}-methylarginine demethylases (RDMs) is controversial [3], this simple and selective method may prove to be a useful tool for the screening of RDMs.

2. Materials and methods

2.1. Reagents and instruments

The peptides were synthesized by GL Biochem., Ltd. (Shanghai, China), with the specified sequence NH_2-cKAKTGAAGKFKRGK-COO^-, where the lowercase cysteine (c) indicates the right-handed amino acid, and the methylation site is at the arginine residue (R).

L-arginine, *NG*-monomethyl-L-arginine, 6-mercapto-1-hexanol (MCH), tris-(2-carboxyethyl) phosphine hydrochloride (TCEP), 8-hydroxyquinoline (8-HQ), and sodium hypochlorite were purchased from Sigma-Aldrich. Electrochemical measurements were carried using a CHI1200C electrochemical analyzer (CH Instruments, USA). The three-electrode system consisted of the peptide-monolayer- modified gold electrode (diameter 3 mm) as the working electrode, a saturated calomel electrode (SCE) as the reference electrode, and a platinum pillar as the counter electrode. Colorimetric measurements were made using a microplate reader (Tecan, Infinite® 200 Pro) and a spectrophotometer (NanoDrop 2000, Thermo).

2.2. Sakaguchi reaction on different arginine substrates

We designed eight sets of samples to test the feasibility of the arginine color change reaction: 1 mM L-arginine (Arg), methylated L-arginine (Arg^{Met}), L-lysine (Lys) and L-glycine (Gly), 0.1 mM methylated peptide (Pep^{Met}), 0.1 mM unmethylated peptide (Pep), and 1 mg mL^{-1} BSA. We took 250 μL of each sample, then added the color reaction system with 125 μL of 10% sodium hydroxide, 12.5 μL of 10 mM 8-HQ and 20 μL of 4% sodium hypochlorite. After mixing, the mixture was incubated at 25 °C for 20 min.

2.3. Electrode modifications

The electrode was prepared as described in our previous reports, with a slight modification [28,29]. The gold electrode (3.0 mm in diameter) was first polished to a smooth, mirror-like surface using 1 μM, 0.3 μM, and 0.05 μM alumina slurries consecutively. The residual alumina powder was then removed by sonicating the electrode sequentially in ethanol and double-distilled water. Afterward, the electrode was immersed in piranha solution (98% H_2SO_4 and 30% H_2O_2, v/v 3:1) for 10 min and washed with double-distilled water. It was then soaked in 50% HNO_3 for 30 min, sonicated in ethanol and ultrapure water for 5 min and electrochemically cleaned in 0.5 M H_2SO_4 by a cyclic voltammetry (CV) scan (0 ~ +1.6 V) until the signal was stable. The residual water beads were blown dry with nitrogen.

Peptide mixtures containing 2.5 μM peptide and 5 mM TCEP were dissolved in 10 mM PBS, pH 7.4. Next, 7 μL of the peptide mixtures were dropped onto the cleaned electrode and inverted for 1 h at 25 °C with an Eppendorf tube covered at the top to avoid air-drying. TCEP was used to prevent the thiol-containing peptides from cross-linking with each other. The peptide-modified electrode was then immersed in 100 μL of MCH solution (1 mM in 10 mM PBS, pH 7.0) for 30 min at 25 °C, and finally the electrode was rinsed repeatedly with double-distilled water to remove non-specifically adsorbed MCH. Peptide mixtures (Pep and Pep^{Met}, total 0.1 mM) containing different concentrations of Pep (0, 0.02, 0.04, 0.06, 0.08, 0.1 mM, respectively) were also tested for calibration purposes.

2.4. Electrochemical measurements

Cyclic voltammetry (CV) and square wave voltammetry (SWV) measurements were performed. CV and electrochemical impedance spectroscopy (EIS) were carried out in an electrolytic solution: 20 mM PB, 10 mM $[Fe(CN)_6]^{3-/4-}$ containing 0.1 M KCl, pH 7.4. The CV parameters were: initial voltage, −0.3 V; final voltage, 0.7 V; scan rate 0.1 V s^{-1}. The SWV was performed in 10 mM PB solution, pH 7.4. The SWV parameters were: initial voltage, 0 V; final voltage, 0.8 V; amplitude, 0.025 V; and frequency, 15 Hz. The EIS experimental parameters were: bias potential, 0.213 V; amplitude, 5 mV; frequency range, 0.1–100 kHz.

2.5. Cell transfection and nucleoprotein extraction

Human breast adenocarcinoma cells (MCF-7) were obtained from the ATCC and were maintained in DMEM containing 10% FBS, 10 units mL^{-1} penicillin and 10 g mL^{-1} streptomycin, 37 °C, 5% CO_2. The cells were sub-cultured and seeded in 6-well plates for treatment with 20 mM AdOx (B6120, APExBIO) upon reaching 80% confluence for 24 h, and then the nuclear protein was extracted. The transient transfection was performed using Exfect 2000 Transfection Reagent (T202–01, Vazyme, China) according to the manufacturer's instructions. Briefly, MCF-7 cells were plated in 6-well plates at 1×10^6 cells per well. For PRMT1 knockdown, ~80% confluent cells were transfected with either 2.5 μg of PRMT1-specific shRNA or non-targeting shRNA as a negative control. For PRMT1 overexpression, cells were transfected with either 2.5 μg of pFlag-CMV4-PRMT1 or pFlag-CMV4 as a negative control. All experiments were performed in triplicate. After 48 h of transfection, cells were harvested for nucleoprotein extraction. We extracted the nuclear proteins using a Nuclear and Cytoplasmic Extraction Kit (CW0199, CWBIO, China). Protein concentration was measured with a BCA Protein Assay Kit (CW0014S500, CWBIO, China) and the protein was stored at −20 °C for vitro methylation assays.

2.6. Real sample analysis for PRMT1 activity

The methylation reaction at the electrode surface was conducted in the following mixture: 10 μg of nuclear lysate containing the PRMT1 protein, 10 μL 10 μM SAM, 100 μL 2 × PRMT1 Reaction Buffer (0.1 M Tris-HCl, 0.01 M $MgCl_2$, 8 mM DTT, pH 8.0). The peptide-modified electrode was incubated in a methylation reaction system for 2 h at 37 °C, then used in SWV experiments.

3. Results and discussions

3.1. Feasibility test of arginine methylation assay

To distinguish methylated from unmethylated arginine, we first tested the principle and feasibility of the assay. It is known that the guanidine group of arginine can selectively react with the reductant agent 8-HQ in alkaline conditions, based on the improved Sakaguchi reaction [26]; however, the selectivity of the reaction towards methylated arginine remains uninvestigated, which is critical for the principle of this assay. To this end, methylated arginine (*NG*-monomethyl-L-arginine, Arg^{Met}) and un-methylated arginine (L-arginine, Arg) were tested in an optimized system to examine the selectivity of the reaction. As shown in Fig. 1a, the reaction mixtures containing unmethylated arginine turned brownish red and UV–vis spectrometry measurements showed a characteristic peak at 510 nm, whereas the methylated arginine remained almost colorless. We observed the same results for an arginine-containing peptide (Fig. 1b). The introduction of amino acids such as L-lysine and L-glycine does not interfere with the assay, suggesting that the system has good selectivity (Fig. 1c). After the Sakaguchi reaction, mixtures of Arg and Pep containing unmethylated arginine show a significant increase in the absorption value at 510 nm, indicating the selectivity of the reaction in distinguishing the methylated state. The data therefore show that the Sakaguchi reaction can be exploited for the detection of methylated arginine.

We noticed that the spectra had a relatively high background, which may affect the limit of detection. In order to increase its sensitivity, an electrochemical analytical technique was introduced into the assay. As shown in Fig. 1d and 1e, the electrode surface was modified with Pep and Pep^{Met} for an electrochemical test. The peptide-modified electrode was incubated with 8-HQ. The guanidine group of arginine reacts covalently with 8-HQ, and an electrochemical response from the reacted 8-HQ is detected. This result indicates the feasibility of the Sakaguchi reaction for electrochemical detection.

3.2. Modification and characterization of the fabricated biosensor

The changes to the peptide immobilized on the electrode surface are revealed by differences in the CV and EIS spectra. Fig. 2 shows the CV and EIS spectra of the bare electrode and of the Pep- and Pep^{Met}-modified electrodes. A pair of characteristic redox peaks observed at the bare electrode can be ascribed to direct electron transfer between the electrochemical indicator ($[Fe(CN)_6]^{3-/4-}$) and the electrode surface (black curve). After immobilization of the peptide onto the electrode surface, the peptide prevents the $[Fe(CN)_6]^{3-/4-}$ from approaching the electrode surface. The resulting decreased redox peak values (dashed and dotted curves) and a significant EIS semi-circle (Fig. 2b) indicate the modification of the electrode with a peptide.

3.3. Optimization of reaction conditions for electrochemical assay

The reaction time with 8-HQ at different reaction concentrations was optimized. We used SWV to study the effect of changing time and concentrations. As the reaction time increases, the peak current increases gradually. When the peptide reacted with 10 mM 8-HQ, the electrochemical signals had a relatively lagged response. After 20 min, the electrochemical signal reached a maximum (Fig. 3). We therefore used 20 min and 1 mM HQ as the optimized reaction conditions in the following experiments.

3.4. Quantitative analysis of arginine methylation

As outlined above, an electrochemical method for the detection of the methylation state of arginine has been established, and this method could be used to study the activity of arginine methylases and demethylases. In order to do this, we quantitatively analyzed arginine methylation by using increasing concentrations of the arginine-containing peptide as described in the Materials and Methods section. We observed that the peak current increases linearly with peptide concentration, allowing the construction of a calibration curve with good correlation statistics (R^2 = 0.993) (Fig. 4).

3.5. Electrochemical assay of PRMT1 activity

We then tested the method by measuring the activity of arginine methylase PRMT1 in an experimental laboratory setting. For this, cell nuclear lysate containing PRMT1 was used as a sample for the electrochemical assay, and the control sample was the untreated cell nuclear extract. In parallel, test samples were transfected with a plasmid that overexpresses PRMT1, a plasmid that can knockdown PRMT1 (shPRMT1), and cells treated with AdOx, an inhibitor of PRMT1 [30]. The peptide-modified electrode incubated with each nuclear lysate was subjected to electrochemical testing. As shown in Fig. 5, the electrochemical signals after treating with shPRMT1 and AdOx were higher than that of the control group. The weaker electrochemical signal output revealed enzyme activity after overexpression of PRMT1 that was higher than that of the control group. Based on this electrochemical assay, the level of arginine methylase activity in the cell can be detected.

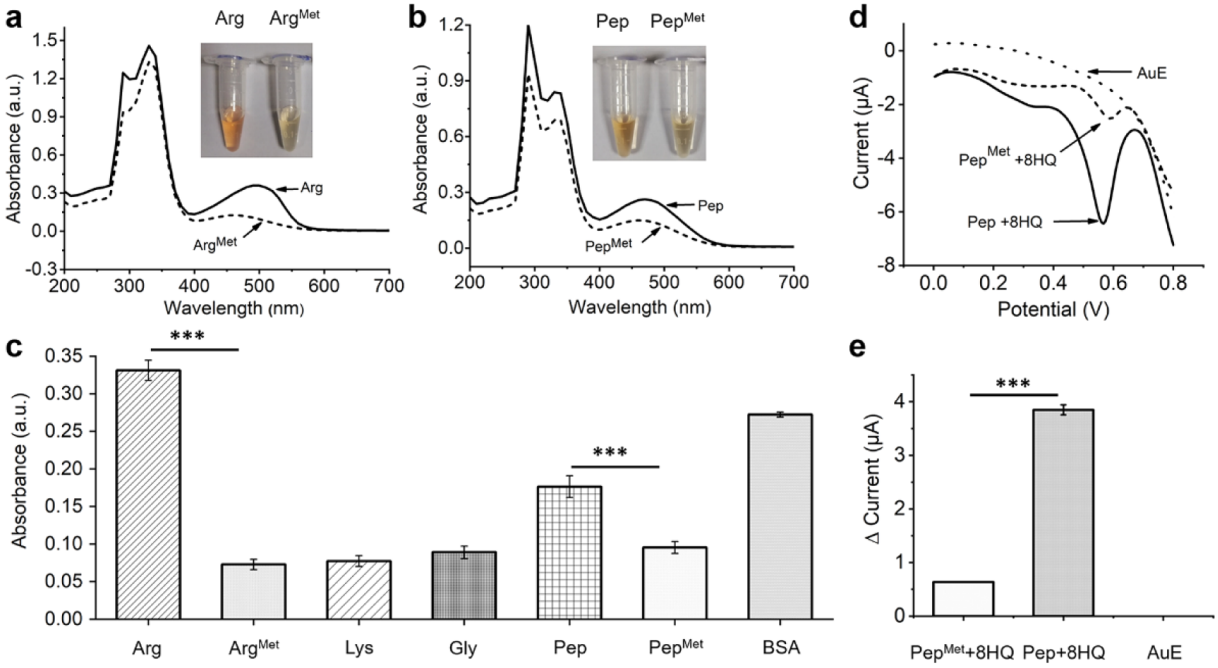

Fig. 1. Principle of the assay. (a) The influence of arginine methylation on the Sakaguchi reaction. The dashed and solid curves show the UV–vis absorption spectra of a reaction mixture containing methylated (Arg^{Met}) and unmethylated arginine (Arg), respectively. (b) The UV–vis absorption spectra of the reaction mixture containing methylated peptide (Pep^{Met}) and unmethylated peptide (Pep). (c) Selective analysis of the assay. 1 mM interfering amino acids (lysine and glycine) and 0.1 mg mL^{-1} BSA were introduced as interfering species and positive controls. The columns show the corresponding absorption values at 510 nm. d. SWVs of the Pep^{Met}- and Pep-modified electrodes after the Sakaguchi reaction. e. Corresponding peak values from the SWV scans. The data show mean ± s.d., $n = 3$.

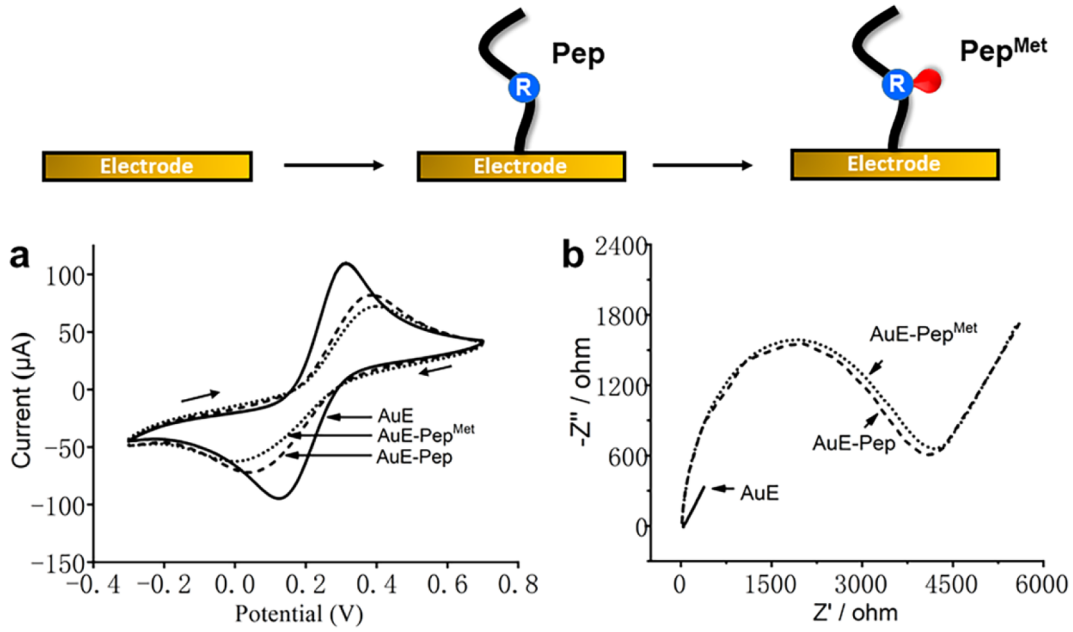

Fig. 2. Characterization of the electrochemical sensor. (a) CVs of the Au electrode (AuE), Pep-modified AuE (AuE-Pep) and Pep^{Met}-modified AuE (AuE-Pep^{Met}). The scan direction is indicated by the arrows. (b) The corresponding EIS spectra. The electrolytic solution used for CV and EIS measurements was 20 mM PB, 10 mM $[Fe(CN)_6]^{3-/4-}$ containing 0.1 M KCl, pH 7.4.

4. Conclusions

To date, the identification of arginine methylation has been challenging. The proposed approach offers a simple technique for detecting arginine methylation and avoids the use of complex labeling or the need to synthesize reporting molecules. We have demonstrated that the developed electrochemical assay method is simple but efficient for detecting protein arginine methylation. The 8-HQ can be covalently linked to the guanidine group of arginine with high selectivity, with the reaction product acting as the signal molecule for electrochemical detection. The use of an electroactive product of the Sakaguchi reaction ensures selective and sensitive detection. Consequently, this electrochemical assay is able to reveal both the methylated and unmethylated states of arginine by tracking the electrochemical responses of the electrode. This study will also be useful for screening PRMT inhibitors, and may allow the identification of possible arginine methylase and demethylase in the future.

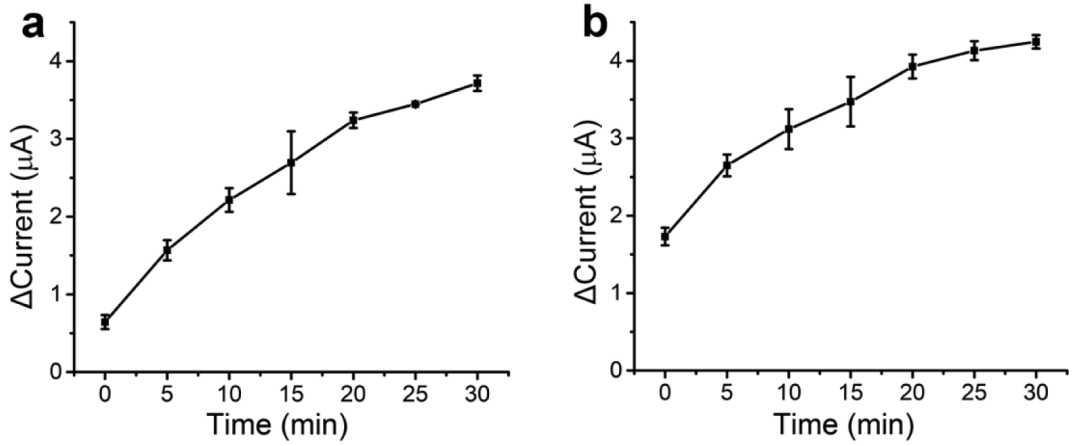

Fig. 3. Optimization of the concentration of 8-HQ and reaction time. Peak current of the SWVs of the Pep-modified electrodes (AuE-Pep) reacted with (a) 1 mM and (b) 10 mM 8-HQ over time. The data show the mean ± s.d., $n = 3$.

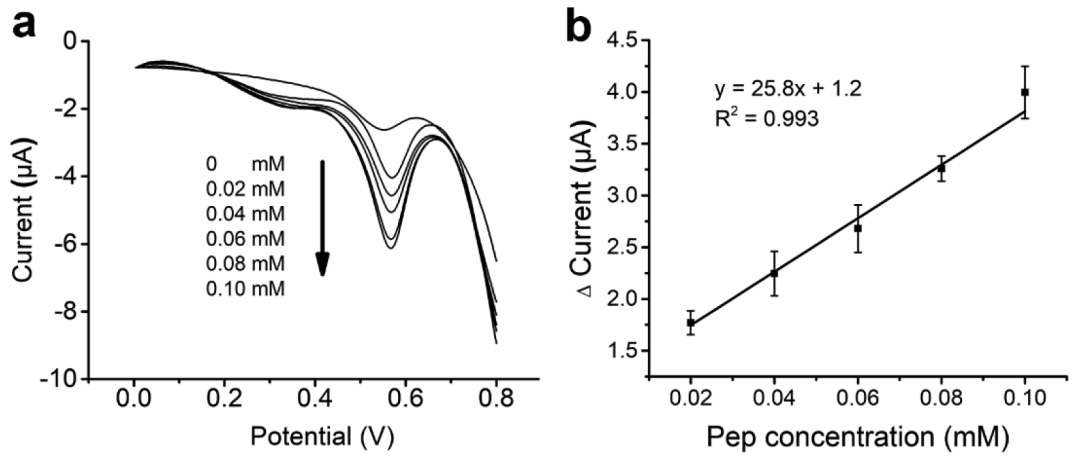

Fig. 4. Quantitative analysis of arginine methylation. The concentrations of arginine-containing peptide used to modify the electrode were 0, 0.02, 0.04, 0.06, 0.08, 0.1 mM, respectively. The data show the mean ± s.d., $n = 3$.

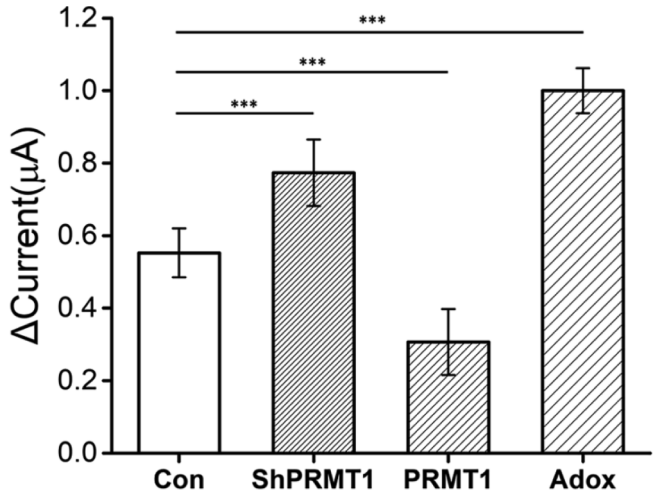

Fig. 5. Electrochemical analysis of arginine methylase PRMT1 activity in real samples. The columns show the electrochemical responses of the control group, and samples treated with ShPRMT1 (a plasmid that knocks down PRMT1), PRMT1 (a plasmid that overexpresses PRMT1), and AdOx (an inhibitor of PRMT1). Error bars indicate the standard deviation for three measurements. ***$p < 0.001$. The data show the mean ± s.d., $n = 3$.

CRediT authorship contribution statement

Mengyuan He: Conceptualization, Methodology, Data curation, Formal analysis, Writing - original draft. **Jiarong Guo:** Methodology, Formal analysis, Software. **Jiahua Yang:** Methodology, Formal analysis, Software. **Yang Yang:** Methodology, Formal analysis, Software. **Songyan Zhao:** Methodology, Formal analysis, Software. **Qiao Xu:** Methodology, Formal analysis, Software. **Tiangxiang Wei:** Methodology, Funding acquisition, Writing - review & editing. **Davide Maria Ferraris:** Writing - review & editing. **Tao Gao:** Conceptualization, Methodology, Project administration, Funding acquisition, Writing - review & editing. **Zhigang Guo:** Project administration, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grant Nos. 81901836, 21705079 and 81872284),

the Natural Science Foundation of Jiangsu Province (Grant Nos. BK20190693, BK20171033 and BE2018714), the Natural Science Foundation of Jiangsu Higher Education Institutions of China (Grant No. 19KJB310004), and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

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