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Identification of glutathione by voltammetric analysis with rolling circle amplification

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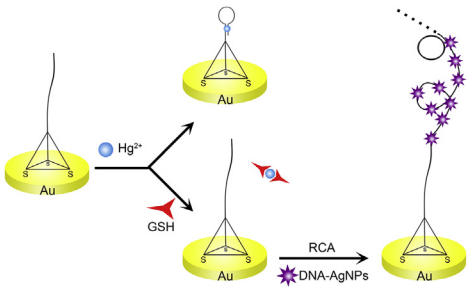
HIGHLIGHTS

- A novel voltammetric biosensor for glutathione detection is fabricated.
- Coordination of Hg^{2+} to glutathione is more favorable than that of T-Hg^{2+}-T.
- Rolling circle amplification product recruits AgNPs for stripping current response.
- The biosensor has a detection limit of 0.1 pM.
- This biosensor performs satisfactorily in real samples.

GRAPHICAL ABSTRACT



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ABSTRACT

Glutathione (GSH), a common tripeptide, plays an essential role in a variety of cellular functions. GSH level is reported to be closely related to human health. In this study, we fabricate an ultrasensitive electrochemical biosensor for GSH quantification. DNA probes are firstly modified on the electrode surface and thymine-Hg^{2+}-thymine is formed. Since GSH is able to chelate Hg^{2+} from the DNA mismatched sites effectively, which leads to DNA structural switching from hairpin to linear strand, rolling circle amplification (RCA) could be initiated with the released linear primer probe. The RCA product with multiple repeating sequences further captures numerous DNA modified silver nanoparticles (AgNPs) by the hybridization of complementary sequences. Stripping voltammetric responses of AgNPs are then detected to reveal GSH concentration. The linear detection range is from 0.1 pM to 10 nM and the limit of detection is 0.1 pM, which is lower than most current analytical methods. This method is also highly selective and functions well against a series of interferents. Additionally, the proposed method has been successfully utilized in human serum samples, which shows fairly good potential in clinical applications.

1. Introduction

Sulfhydryl groups are naturally present in animals, plants and microscopic forms of life, which have a variety of functions like sequestering metal ions and quenching reactive oxygen species [1,2]. Glutathione (GSH), also named as L-γ-glutamyl-L-cysteinylglycine, is one of the most abundant biothiols and endogenous antioxidants, which plays essential roles in cellular defense against inimical heavy metals and energetic free radicals [3,4]. Numerous studies have established relationships between the concentrations of this tripeptide in cells or blood plasma with human health conditions. Blood GSH level is reported to be able to reflect the redox status and can be considered as a useful disease indicator [5]. Abnormal levels of GSH are closely associated with

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different human disorders such as heart disease, liver damage, age-related hearing loss, Parkinson's disease, and cancer [6–10]. Therefore, development of sensitive methods to determine the presence or concentration of GSH is urgently needed [11].

Despite the availability of different techniques for GSH detection, including high-performance liquid chromatography (HPLC) [12], mass spectrometry [13], and fluorescence-based methodology [14,15], several disadvantages are suffered, which may limit their wide applications. These methods may require sophisticated equipment, expensive fluorescence probe and well-trained personnel. On the contrary, electrochemistry-based methodology benefits from merits of rapid response, low cost, convenient operation, high sensitivity and selectivity, which is suited to the detection of GSH [16–20]. For example, Oztekin et al. prepared a poly-*m*-aminophenol modified glassy carbon electrode for electroanalytical determination of GSH [21]. Ru et al. employed multi-walled carbon nanotubes/ubiquinone/ionic liquid nanocomposite as a transducer for GSH sensing [22]. Zhad et al. developed an electrochemical "signal-on" sensor based on the coordination of GSH and mercury ions (Hg^{2+}) that are bound to DNA probe on electrode surface [23]. Areias et al. reported a voltammetric sensor in which GSH forms a 1:1 complex compound with copper ions (Cu^{2+}) for characteristic electrochemical oxidation [24].

In this report, we present a novel electrochemical approach for GSH analysis with simple modification design and high sensitivity. Rolling circle amplification (RCA) is initiated after DNA structural switching on the electrode surface, which is induced by GSH mediated Hg^{2+} displacement from thymine-Hg^{2+}-thymine. Further attached silver nanoparticles (AgNPs) by DNA hybridization provide significant silver stripping signals, which not only reveal electrochemical behaviors during different modification stages, but also provide quantitative information of GSH concentration.

2. Experimental

2.1. Materials and chemicals

GSH, trisodium citrate, silver nitrate ($AgNO_3$), mercury nitrate ($Hg(NO_3)_2$), sodium borohydride ($NaBH_4$), tris(2-carboxyethyl) phosphine hydrochloride (TCEP) and hexaammineruthenium(III) chloride ($[Ru(NH_3)_6]^{3+}$) were ordered from Sigma (USA). Phi29 DNA polymerase and T4 DNA ligase were purchased from New England Biolabs Ltd. (Beijing, China). Human serum samples were supplied by local hospital (Suzhou, China). The other reagents were of analytical grade and were used as received. All solutions were prepared with water that was purified from a Millipore system. DNA oligonucleotides were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The sequences were listed in Table 1. The underlined bases of tetrahedron A constitute thymine-thymine mismatches.

2.2. Instrumentation

All electrochemical experiments were carried out on a CHI 660D electrochemical workstation (CHI instruments, Shanghai, China). Traditional three electrode system was applied, which contained platinum wire auxiliary electrode, Ag/AgCl reference electrode and gold working electrode. After the modification steps of working electrode, linear sweep voltammetry (LSV) experiments were performed in the electrolyte of 0.1 M KCl with the scan rate of 0.1 V s^{-1}. Chronocoulometry (CC) experiments were performed in 10 mM Tris-HCl buffer solution containing 50 μM $[Ru(NH_3)_6]^{3+}$ with the pulse period of 250 ms.

2.3. DNA tetrahedron formation

DNA tetrahedron that was used to modify the electrode was prepared as follows. Four single-stranded DNA solutions (tetrahedron A, B, C, and D) were firstly prepared in 10 mM Tris-HCl buffer with 10 mM TCEP, 50 mM $MgCl_2$ (pH 8.0). The final concentrations were 4 μM. Next, the four solutions were blended with the ratio of 1: 1: 1: 1. The mixture (DNA tetrahedron solution) was then heated to 95 °C for 2 min, which was cooled to room temperature for further use.

2.4. Electrode pretreatment and modification

The gold electrode (2 mm) was incubated with piranha solution (98% H_2SO_4: 30% H_2O_2 = 3: 1) for 5 min (*Caution: Piranha solution reacts violently with organic solvents and should be handled with great care!*). Next, it was polished to a mirror-like surface with P5000 sand paper and then 1, 0.3, 0.05 μm alumina slurry, respectively. The electrode was cleaned by ultrasonication for 5 min in ethanol and then in double-distilled water. Subsequently, it was immersed in 50% HNO_3 for 0.5 h and then electrochemically cleaned with 0.5 M H_2SO_4 for 20 cycles to remove any remaining impurities. After that, the electrode was dried with nitrogen and was incubated with DNA tetrahedron solution for 8 h.

2.5. Preparation of DNA-AgNPs conjugates

Synthesis of bare AgNPs was carried out by the borohydride reduction of $AgNO_3$. Briefly, 0.25 mM $AgNO_3$ and trisodium citrate solution was prepared, which was then blended with 10 mM $NaBH_4$ solution. The volumes were 100 mL and 3 mL, respectively. The mixture was under violent stirring for 0.5 h and then left to sit overnight in the dark. Yellow colored and transparent AgNPs solution was thus synthesized, which was then purified by three cycles of centrifugation at 12000*g* for 0.5 h. The as-prepared AgNPs solution was further interacted with 10 μM DNA signal probe for 24 h to achieve silver-amino binding. Finally, DNA-AgNPs conjugates were purified by centrifugation procedure.

2.6. Electrochemical detection of GSH

For the formation of hairpin structure on top of DNA tetrahedron at the electrode surface, the electrode was incubated with 15 nM Hg^{2+} for 1 h. Hg^{2+} was dissolved in H_2O directly and pH was

Table 1
DNA sequences used in this work.

Name	Sequence (from 5′ to 3′)
Tetrahedron A	ACATTCCTAAGTCTGAAACATTACAGCTTGCTACACGAGAAGAGCCGCCATAGTACCGTTTGGGTTTTTAATCCCTATAAATACCCTAAC
Tetrahedron B	SH-$(CH_2)_6$-TATCACCAGGCAGTTGACAGTGTAGCAAGCTGTAATAGATGCGAGGGTCCAATAC
Tetrahedron C	SH-$(CH_2)_6$-TCAACTGCCTGGTGATAAAACGACACTACGTGGGAATCTACTATGGCGGCTCTTC
Tetrahedron D	SH-$(CH_2)_6$-TTCAGACTTAGGAATGTGCTTCCCACGTAGTGTCGTTTGTATTGGACCCTCGCAT
Padlock probe	phosphoryl-TTATAGGGATTCTCTATCTGTTAGGGTAT
Signal probe	NH_2-$(CH_2)_6$-GATTCTCTATCT

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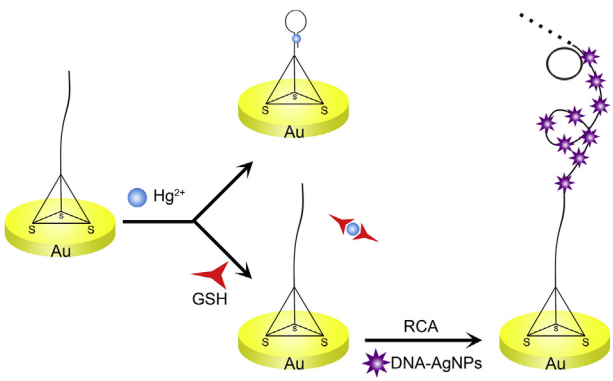
adjusted to be 5.0 to avoid the formation of HgO. Subsequently, the electrode was immersed in standard GSH solutions with a series of concentrations for another 1 h. Afterward, the electrode was rinsed with double-distilled water and then immersed in 50 mM Tris-HCl buffer solution containing 1 μM DNA padlock probe, 5 unit mL^{-1} T4 DNA ligase, 10 mM $MgCl_2$, 10 mM DL-Dithiothreitol (DTT), 1 mM adenosine triphosphate (ATP) at 22 °C for 1 h. Later, the electrode was further incubated with 50 mM Tris-HCl buffer solution containing 50 unit mL^{-1} phi29 DNA polymerase, 10 mM $MgCl_2$, 4 mM DTT, 10 mM $(NH_4)SO_4$, 0.2 mg mL^{-1} bovine serum albumin (BSA), 0.5 mM deoxy-ribonucleotide triphosphate (dNTP) at 37 °C for 1 h. After that, the electrode was treated with DNA-AgNPs conjugates for another 1 h, and was then electrochemically measured.

To evaluate the selectivity of the proposed GSH biosensor, Na^+, K^+, glutamine, urea, glucose, BSA and cysteine solutions with certain concentrations were prepared separately, which were then used to interact with DNA tetrahedron and Hg^{2+} modified electrode instead of GSH. To further check the practical utility of the method, diluted human serum samples with spiked GSH were used instead of standard GSH solutions, which were electrochemically detected after rolling circle amplification and DNA-AgNPs conjugates treatment.

3. Results and discussion

3.1. Sensing principle

The biosensor is simply constructed on the gold electrode interface coupling GSH mediated DNA structural switching and subsequent RCA. As depicted in Scheme 1, DNA tetrahedron is formed through the hybridization between four single-stranded DNA and is then attached on the gold electrode through the thiols on the three vertexes [25–27]. Since the pendant linear sequence on top of DNA tetrahedron contains thymine-thymine mismatches, hairpin structure is constructed through special interaction between Hg^{2+} and mismatched sites. GSH could chelate Hg^{2+} depending on two or more sites including thiol and carboxy groups. Since the binding of GSH and Hg^{2+} is more favorable than that of thymine-Hg^{2+}-thymine complex, hairpin structure is destroyed and pendant linear sequence is restored, which initiates subsequent RCA with the assistance of circular DNA template and DNA polymerase [28]. Long single-stranded RCA product with multiple repeating sequences are complementary with those of DNA-AgNPs conjugates. Therefore, numerous AgNPs could be located on the electrode surface, which provide significant silver stripping signals [29]. The recorded electrochemical response is associated with GSH induced DNA structural switching, and quantitative determination of GSH is achieved.



Scheme 1. Scheme of the electrochemical biosensor for GSH assay.

3.2. Electrochemical study of the biosensor

The surface density of DNA tetrahedron is 0.92 pmol cm^{-2}, obtained by CC measurements of $[Ru(NH_3)_6]^{3+}$ and calculation according to Cottrell equation (Fig. S1) [30]. The amount of $[Ru(NH_3)_6]^{3+}$ is firstly obtained from the equation $\Gamma = Q/nAF$, which is then converted to the surface density of DNA tetrahedron (Γ: electrode surface coverage density, Q: difference between chronocoulometric intercepts for the experiments in the presence and absence of DNA tetrahedron, n: the number of electrons transferred in one redox reaction, A: electrode surface area, F: Faraday constant). Electrode surface modification is characterized by LSV. As depicted in Fig. 1, no silver stripping peak can be observed on DNA tetrahedron modified electrode after incubation with Hg^{2+} (curve a). Since thymine-Hg^{2+}-thymine forms and the DNA strand on top of DNA tetrahedron is transformed to a hairpin structure, RCA lacks linear primer initiation, thus DNA-AgNPs cannot be effectively located on the surface of the electrode. A negligible peak appears due to nonspecific adsorption of AgNPs (curve b). However, the added GSH can effectively chelate Hg^{2+}, displacing the ions from the DNA probe, which returns to the single-stranded form and

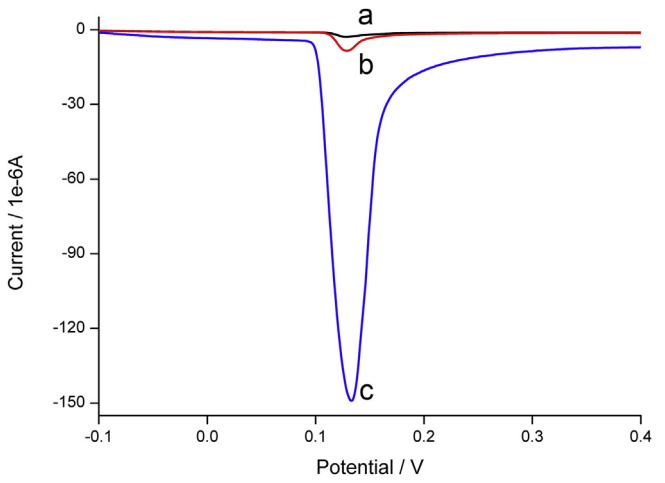


Fig. 1. LSV responses obtained at (a) DNA tetrahedron modified electrode after incubation with Hg^{2+}, (b) without and (c) with coordination of Hg^{2+} to GSH and further RCA coupled with AgNPs attachment.

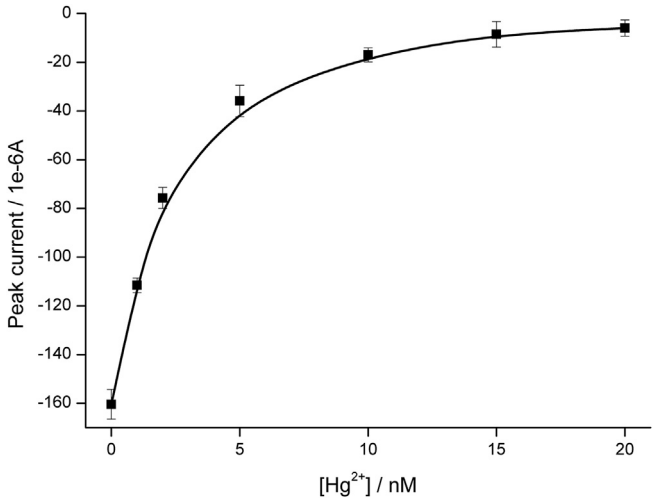


Fig. 2. Optimization of Hg^{2+} concentration to block RCA and subsequent AgNPs localization.

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triggers the subsequent RCA and DNA-AgNPs attachment. Numerous located AgNPs can be electrochemically measured and a significant silver stripping peak is observed (curve c).

3.3. Voltammetric analysis of GSH

Optimization experiments show that DNA structural switching from single strand to hairpin is Hg^{2+} concentration-dependent. As shown in Fig. 2, LSV peak current increases gradually with more Hg^{2+}. 15 nM Hg^{2+} is chosen for further experiments since the obtained electrochemical signal is plateaued with the concentration. We have conducted more detailed experiments to investigate the voltammetric responses with different amount of GSH. As shown in Fig. 3, LSV peak current is found to be continuously rise with the increase of GSH level, which is due to the release of larger amount of Hg^{2+} by more GSH chelating and the resulted RCA to assemble numerous AgNPs. Direct relationship between the peak current generated from AgNPs and the logarithm of GSH concentration is studied. A linear fit in the range from 0.1 pM to 10 nM is revealed in Fig. 4 with the regression equation as:

$$i(\mu A) = -377.574 - 28.214 \times \lg c(M), R^2 = 0.991$$

The limit of detection (LOD) is calculated to be 0.1 pM [31]. Table 2 displays comparisons of the results for GSH measurement obtained from the proposed method and those from recently reported biosensors. LOD of this work is comparable or even better than that from the other methods. Linear detection range is the widest, which covers five orders of magnitude. The response time after the employment of samples to be tested is about 4 h due to the procedures of DNA ligation and RCA. DNA tetrahedron used to modify the electrode interface provides excellent molecule layer, which enhances the efficiency of molecular recognition and helps avoid the procedure of spacer molecules modulation. The modified electrode displays high stability, which can be used for GSH quantification with high accuracy after stored in 4 °C for 1 month. Moreover, different working electrodes are modified in the same manner and GSH levels are then detected. Variation coefficients for three duplicate measurements are calculated. The average relative standard deviation (RSD) is 4.97%, demonstrating that this

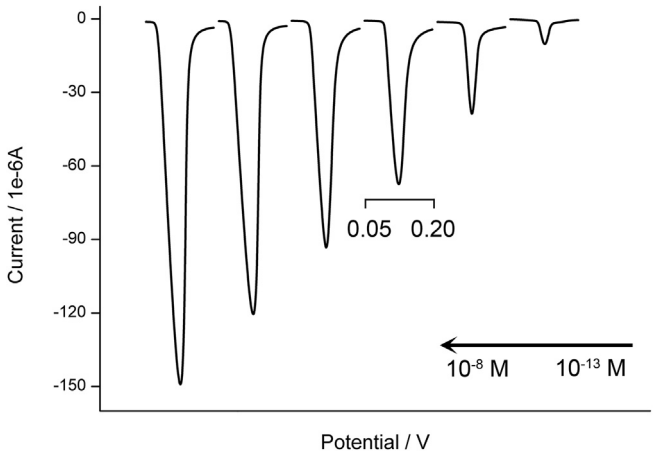

Fig. 3. LSV responses obtained with different concentrations of GSH: 10^{-13}, 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8} M (from right to left).

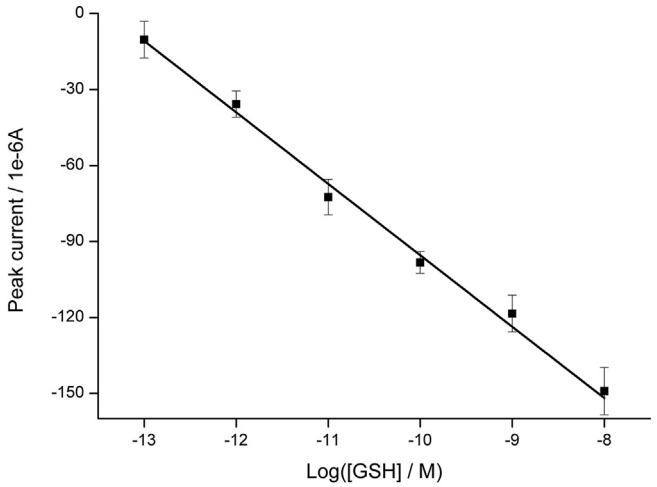

Fig. 4. A quantitative linear correlation between LSV peak current and GSH concentration over a wide range from 10^{-13} to 10^{-8} M.

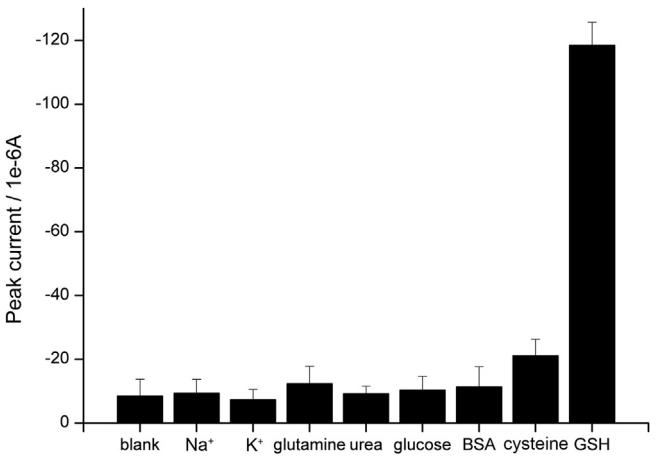

Fig. 5. Selectivity investigation of the proposed method for GSH assay against some interferents (1 nM).

Table 2
Comparison of the proposed GSH biosensor with other existing methods.

Strategies	Linear range (M)	LOD (M)	Ref.
Monochlorinated BODIPY	0 to 6×10^{-5}	8.6×10^{-8}	[34]
electrochemiluminescence amplification of carbon dots	10^{-7} to 10^{-6}	5.43×10^{-8}	[35]
FRET-based carbon dots–MnO_2 nanosheet	2×10^{-7} to 6×10^{-4}	2.2×10^{-8}	[36]
Dispersion-dominated colorimetric sensor	2.5×10^{-8} to 3.75×10^{-7}	1.09×10^{-8}	[37]
Two-photon excitation, upconversion luminescent quantum dots	10^{-8} to 10^{-5}	6.5×10^{-9}	[38]
Fe_3O_4@PANI/rGO nanocomposites	5×10^{-8} to 5×10^{-5}	3×10^{-9}	[39]
Hg^{2+}-mediated strand displacement reaction	5×10^{-10} to 5×10^{-6}	1.4×10^{-10}	[40]
DNA displacement and gold nanoparticles	10^{-12} to 10^{-10}	4×10^{-13}	[41]
GSH-Hg^{2+} coordination and RCA	10^{-13} to 10^{-8}	10^{-13}	This work

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Table 3
Determination of GSH in diluted serum samples.

Samples	Spiked (nM)	Detected (nM)	RSD (%)
1	1.00	1.03	3.00
2	2.00	2.15	7.50
3	5.00	5.24	4.80
4	10.00	10.33	3.3

approach could be well reproduced, which is superior to other methods with relatively poor reproducibility.

3.4. Specificity investigation

To verify the high selectivity of the detection of GSH, control molecules including ions, amino acids and proteins are tested. As shown in Fig. 5, the electrochemical signal intensities responding to these control molecules are much lower than that of GSH, demonstrating that the method responds only to GSH and interference arising from other molecules is insignificant. Since the binding affinity of GSH is much higher than that of cysteine toward Hg^{2+}, the design of GSH mediated Hg^{2+} displacement enables the proposed method to distinguish GSH from other small thiol species like cysteine, while some previously reported methods cannot [32,33]. Moreover, we have applied this method in complex biological real samples. Diluted human serum samples are spiked with different amount GSH. The above real samples are then measured. Satisfactory results reconfirm the high selectivity of this method and suggest that the method has great potential to be applied for clinical use (Table 3).

4. Conclusion

In conclusion, we demonstrate a voltammetric biosensor for ultrasensitive detection of GSH. This novel strategy is based on DNA structural switching responsive to the chelation of GSH. RCA can then be realized on the electrode surface, whose products capture numerous AgNPs by hybridization between complementary sequences. By tracing the stripping voltammetric response of AgNPs, initial GSH level can be determined. The resultant biosensor is proven to be highly sensitive with the LOD as low as 0.1 pM. Moreover, its specificity for GSH analysis is also high, which can effectively discriminate target molecules in complex biological samples. The proposed method may provide a simple but powerful tool for GSH monitoring in physiological or pathological condition, which has clinical significance in the diagnosis and therapy of certain diseases.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.aca.2016.09.035.

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