[Frontiers in Bioscience 13, 3768-3774, May 1, 2008]

Voltammetric investigation of surface-confined proteins

Yuan Yuan, Xu Zhu, Jianxiu Wang

College of Chemistry and Chemical Engineering, Central South University, Changsha, Hunan, 410083, P. R. China

TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Experimental
3.1. Reagents
3.2. Instruments and electrodes
3.3. Procedures
3.4. Electrochemical detection
4. Results and discussion
4.1. Voltammetric behavior of Fc-SH linked to surface-confined lysozyme
4.2. Voltammetric behavior of Fc-SH linked to surface-confined MT
5. Conclusions and perspectives
6. Acknowledgements
7. References

1. ABSTRACT

Development of sensitive and selective methods to detect proteins at trace levels is of great biological importance. Via derivatization with a bifunctional cross-linker 4-maleimidobutyric acid N-hydroxysuccinimide ester (GMBS) and an electrochemical marker 11-ferrocenyl-1-undecanethiol (Fc-SH), voltammetric determination of surface-confined proteins electrostatically adsorbed onto the polyelectrolyte of poly (sodium 4-styrenesulfonate) (PSS) or poly(allylamine hydrochloride) (PAH)-covered surfaces could be realized. The utilization of PSS or PAH was anticipated to reduce the nonspecific adsorption of the proteins on the surface. Two kinds of proteins with no redox activity or exhibiting complex or ill-defined voltammetric peak(s), i.e. the positively charged lysozyme and negatively charged metallothionein (MT) were demonstrated. Due to the incorporation of the bifunctional reagent GMBS and the redox active Fc groups onto the protein-modified electrodes, well-defined voltammetric peaks of high signal intensity were obtained. The anodic peak heights were found to be dependent on the surface density of the proteins electrostatically binded to the polyelectrolyte-coated surface. The present method can measure lysozyme concentration as low as 0.1 nM.

2. INTRODUCTION

The development of sensitive and selective methods to detect proteins at trace levels is currently more desirable. For example, proteomics research requires the facile detection of signaling proteins due largely to the influence of the cellular stimulus on their contents (1). Similarly, in medical diagnostics, it is important to determine the contents of the characteristic proteins (2). Of the proteins studied so far, lysozyme and metallothionein (MT) are two important ones. It is commonly known that lysozyme is a single chain-protein with a molecular weight of 14.6 kDa found in egg white, tears, and other secretions. It exerts its function against infection via breaking down the polysaccharide walls of many kinds of bacterias (3). The determination of lysozyme can find its potential applications in clinical diagnosis, toxicological research and is of great significance for investigation of the influence of environmental pollution on human health (4). MT belongs to a class of metal-binding proteins composed of a large number of cysteine residues (5-7). Due to its strong metal-binding affinity, MT was supposed to play an important role in regulation of essential metals and detoxification of heavy metals (8). Determination of MT is of great importance in environmental toxicology.

Thus far, a great number of methods have been utilized for the quantitative detection of these proteins. These methods include but are not limited to surface plasmon resonance (9), fluorescence spectroscopy (10), surface-enhanced raman spectroscopy (11), attenuated total reflection Fourier transform infrared spectroscopy (12), enzyme-linked immunosorbent assay (ELISA) (13), radioimmunoassay (RIA) (13), and atomic spectroscopic techniques (14). However, these methods either involve sophisticated instrumentation, or have certain limitations (e.g., photobleaching in fluorescence detection (15, 16) and problems associated with the use of radioisotopes and enzymes in RIA and ELISA, respectively).

Voltammetric techniques are simple, sensitive, and inexpensive to implement (17). Nevertheless, for the two proteins mentioned above, lysozyme exhibits no redox activity (18) and in the case of MT, very small or no redox waves were generally observed at solid electrodes (19, 20). Conceivably, the introduction of an electroactive label should allow biomolecules that are either electro-inactive or do not exhibit reversible voltammetric responses to be detected. As demonstrated by Hill and Kraatz *et al.*, labeling biomolecules with electroactive labels has become an attractive avenue in bioanalysis (21-23). Several papers reported the electrochemical determination of peptides or proteins bearing an electrochemical tag in solution (21, 22, 24). However, due to the limitations such as sophisticated sample pretreatment and much sample requirement in these methods, determination of biomolecules affixed onto surfaces is becoming increasingly important in view of the development and characterization of heterogeneous biosensors and microarrays (25).

In this paper we describe a novel method using a bifunctional cross-linker GMBS and an electrochemical marker Fc-SH to detect two kinds of surface-bound proteins, the positively charged lysozyme and the negatively charged MT. Lysozyme and MT were electrostatically adsorbed onto the polyelectrolytes of PSS and PAH-coated surfaces, respectively. The utilization of PSS and PAH to electrostatically bind the proteins and to reduce the background signals was demonstrated (26). GMBS can be used for the subsequent cross-linking of the functional groups of the proteins (27, 28). This technique allows voltammetric determination of surface-confined proteins at trace levels.

3. MATERIALS AND METHODS

3.1. Reagents

Cysteamine hydrochloride, 11-mercaptoundecanoic acid (MUA), poly (sodium 4-styrenesulfonate) (PSS), poly(allylamine hydrochloride) (PAH) and 4-maleimidobutyric acid N-hydroxysuccinimide ester (GMBS) were acquired from Sigma-Aldrich. 11-ferrocenyl-1-undecanethiol (Fc-SH) was obtained from Dojindo (Japan). Egg white lysozyme was purchased from Amresco. MT was obtained from Hunan Weih-Lugu Biotech Co., Ltd (China). *N*-(2-ethyl-ferrocene)maleimide (Fc-Mi) was synthesized according to literature procedures (22). All other chemicals were of reagent grade. All solutions were prepared with deionized water treated with a water purification system (Simplicity 185, Millipore Corp.).

3.2. Instruments and electrodes

Electrochemical experiments were conducted with a CHI 660B electrochemical workstation (CH Instruments, Austin, TX) in a conventional three-electrode cell. The gold working electrodes have a diameter of 2 mm. A platinum wire and a Ag/AgCl electrode were used as the auxiliary and the reference electrodes, respectively. Prior to each measurement, the Au electrodes were polished with diamond pastes and alumina slurry down to 0.3μm on a polishing cloth (Buehler, Lake Bluff, IL), followed by sonicating in water. The supporting electrolyte was a 0.1 M $KClO_4$ solution.

3.3. Procedures

Cysteamine hydrochloride SAMs were first formed by immersing the Au electrodes into a water solution containing 4.0 mM cysteamine hydrochloride overnight. The electrodes were then washed thoroughly with deionized water. The resulting cysteamine hydrochloride SAMs were positively charged. For electrostatic adsorption, 10μL water solution containing 4mg/mL negatively charged PSS was cast onto the electrodes for 30 min. These electrodes were then washed with water and followed by casting phosphate buffered saline (PBS, 10.0 mM phosphate/137 mM NaCl, pH = 7.4) comprising a given concentration of lysozyme onto the electrodes. Lysozyme, being positively charged at pH 7.4 (PI=11.1) (29, 30), was adsorbed onto the PSS-covered electrodes via the electrostatic interaction and the above reactions were allowed to proceed for 30 min in a humidified Styrofoam chamber. After the surface was thoroughly rinsed with a washing buffer, another 10 μL aliquots of 4.0 mM GMBS solution in DMSO were cast onto the electrodes. The cross-linking reaction between the N-hydroxysuccinimide esters of GMBS and the primary amine groups of lysozyme to form an amide bond can be realized. GMBS, being a bifunctional cross-linking reagent, can be used further for Fc-SH attachment. The maleimide groups situated at the solution termini of the SAMs were then immersed into a 4.0 mM Fc-SH solution dissolved in ethanol for about 20 h. The double bond of the maleimide groups undergoes an alkylation reaction (Michael-type electrophilic addition) by forming a stable thioether bond with sulfhydryl groups of Fc-SH (31). The reaction has been shown to be several orders of magnitude faster than that between the maleimide groups with amino or hydroxyl groups (32). This step was followed by an extensive wash with ethanol and water to rid any possible nonspecifically adsorbed Fc-SH off the electrodes.

For electrochemical determination of negatively charged proteins, MUA SAMs were first formed by immersing the Au electrodes into 4.0 mM MUA solution dissolved in ethanol overnight. The electrodes were then washed thoroughly with ethanol and deionized water. The resulting negatively charged MUA SAMs were used for electrostatic adsorption of positively charged PAH. MT, being negatively charged (PI=4.2) at PBS solution (pH 7.4)

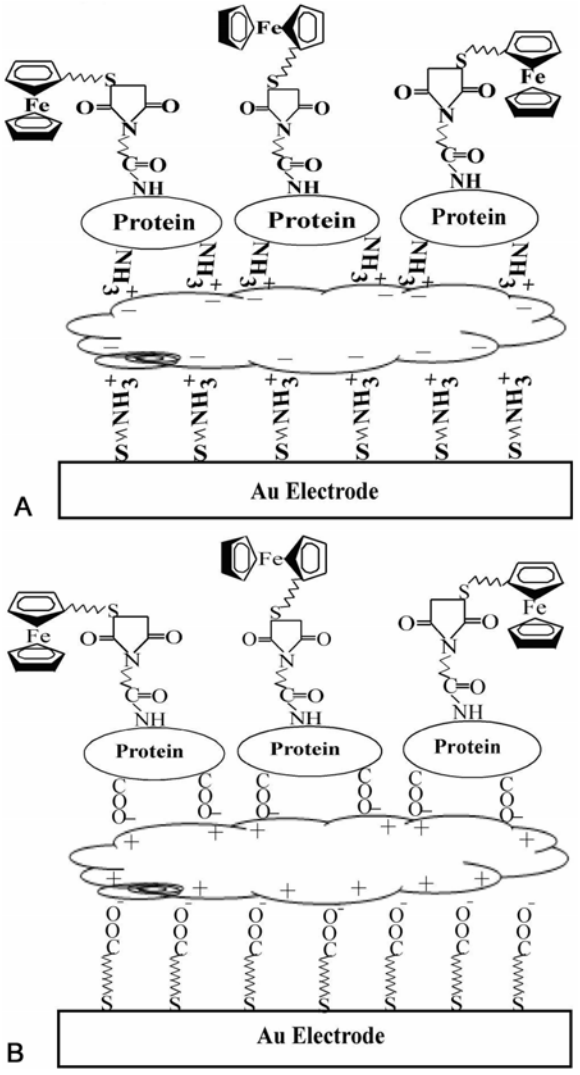

Figure 1. Schematic representation of the voltammetric detection of positively charged (a) and negatively charged (b) proteins with no redox activity or exhibiting complex or ill-defined voltammetric peak(s) via oxidation of the ferrocene moieties. For clarity, PSS, PAH, protein, and alkanethiol molecules are not drawn to scale.

(33), was adsorbed onto the PAH covered-electrode via electrostatic interaction. In a similar step, the primary amine groups in MT were derivatized with GMBS for the attachment of Fc groups. For comparison, Fc-Mi was attached to the cysteine residues on the MT molecules through a Michael-type electrophilic addition reaction (31).

3.4. Electrochemical detection

Once the electrochemical marker Fc-SH had been attached onto the electrode surface, the electrode was transferred to a 0.1 M KClO4 solution and characterized voltammetrically with the potential range between 0 and 0.7 V. All electrochemical experiments were conducted at the ambient temperature.

4. RESULTS AND DISCUSSION

Figure 1 illustrates the principle behind the voltammetric detection of positively charged (a) and negatively charged (b) proteins with no redox activity or exhibiting complex or ill-defined voltammetric peak(s) via oxidation of the ferrocene moieties. In Figure 1a, positively charged cysteamine hydrochloride SAMs were first formed on the gold surface. Next, negatively charged PSS and positively charged lysozyme molecules were adsorbed onto the cysteamine hydrochloride SAMs alternately via the electrostatic interaction. While in Figure 1b, PAH and negatively charged MT were adsorbed onto the MUA SAMs alternately. In both cases, the primary amine groups of lysozyme or MT were derivatized with the bifunctional reagent GMBS. The cross-linking reaction between the N-hydroxysuccinimide esters of GMBS and the primary amine groups of lysozyme or MT to form an amide bond can be accomplished. The maleimide groups situated at the solution termini of the SAMs were further derivatized with Fc-SH. The number of Fc-SH groups attached might be dependent on the structure and surface coverage of the immobilized biomolecules. Consequently, only the Fc-SH groups that are positioned in close vicinity of the electrode are accessible for facile electron transfer reactions.

4.1. Voltammetric behavior of Fc-SH linked to surface-confined lysozyme

Curve a in Figure 2 is a cyclic voltammogram (CV) acquired at electrodes covered with cysteamine hydrochloride and then PSS after electrostatic adsorption of lysozyme, followed by derivatization with GMBS, and the subsequent Fc-SH attachment. A pair of well-defined redox peaks, with the anodic peak at 0.403 V and the peak potential separation (ΔEp) of 0.024 V at a scan rate of 0.05 V s-1 was observed. CVs obtained at scan rates between 0.05 and 1 V s-1 were shown in Figure 3, and the plot of anodic peak currents (Ipa) against the scan rates ranging from 0.05 to 1 V s-1 was linear with a correlation coefficient of 0.9989 (the inset of Figure 3), suggesting that Fc-SH was surface-confined (17). For comparison, the same procedure was implemented in which PSS was replaced by PAH (curve b), no distinct voltammetric peaks were observed. We should note that the surface-confined cysteamine hydrochloride SAMs and PAH take the same charge and they will repulse each other. As a result, the positively charged lysozyme molecules can not be adsorbed onto the electrode surface. We also conducted an experiment in which the electrode was modified with cysteamine hydrochloride and then PSS, but without having exposed the electrode to the lysozyme solution (curve c). The absence of any discernable CV waves in curve c indicates that the Fc moieties do not nonspecifically adsorb onto the electrode surface. A third control experiment in which the attachment of Fc-SH onto the GMBS cover-electrode was replaced by Fc was carried out, no electrochemical response could be obtained (data not shown). This is conceivable that the maleimide groups situated at the solution termini of the SAMs undergo an alkylation reaction with sulfhydryl groups of Fc-SH. The control CV was also acquired at an electrode modified with cysteamine hydrochloride after adsorption of

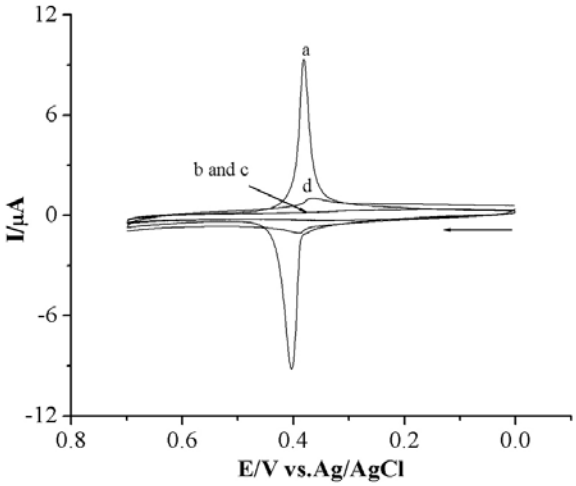

Figure 2. CVs acquired at electrodes covered with cysteamine hydrochloride and polyelectrolyte in the case of PSS (a) or PAH (b) after electrostatic adsorption of lysozyme from PBS solution comprising 50 μM lysozyme, followed by derivatization with GMBS, and the subsequent Fc-SH attachment. The electrochemical response acquired at electrodes covered with PSS in the absence of lysozyme, followed by other subsequent surface modifications is shown as curve c, and curve d was obtained in the absence of PSS. The scan rate was 0.05 V s^{-1}, and 0.1 M $KClO_4$ was used as the electrolyte solution. The arrow indicates the scan direction.

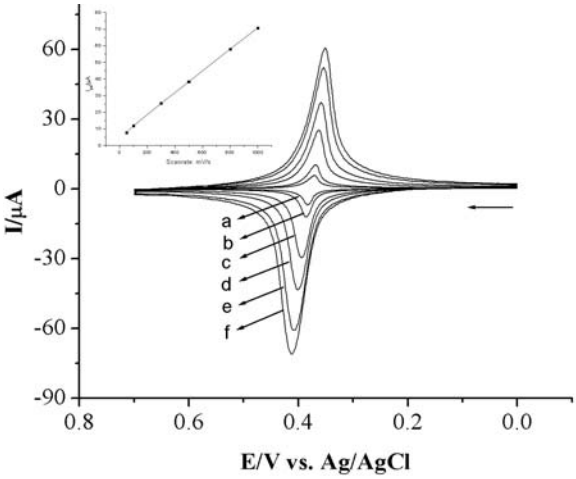

Figure 3. CVs acquired at electrodes covered with cysteamine hydrochloride and PSS after electrostatic adsorption of lysozyme, followed by derivatization with GMBS, and the subsequent Fc-SH attachment at scan rates of a) 0.05, b) 0.1, c) 0.3, d) 0.5, e) 0.8, f) 1 V s^{-1}. The concentration of lysozyme used is 50 μM. The inset shows the plot of anodic peak currents (I_{pa}) against the scan rates. Other experimental conditions are the same as those in Figure 2.

lysozyme, followed by other subsequent surface modifications (curve d). In comparison with curve a, a pair of much smaller voltammetric peaks originating from nonspecific adsorption of Fc tags was obtained. The utilization of polyelectrolytes could eliminate the nonspecific adsorption of the proteins and reduce the background signals. This is in accordance with the literature reported (26). It can be seen from curve a, the voltammogram exhibits a characteristic diffusional "tailing" of the peaks. We have attributed the diffusional tailing to the repulsion among the positively charged ferrocenium moieties confined at the electrode surface (34). It is commonly known that most of the proteins are negatively charged at physiological conditions, the present protocol for the determination of positively charged proteins is becoming increasingly important in view of the development of heterogeneous biosensors.

We then estimated the sensitivity and dynamic range of the method. The dependence of the Fc oxidation peak current on the concentration of lysozyme used for its immobilization is shown in Figure 4. The peak was still discernable even when as little as 0.1 nM of lysozyme was analyzed. The percent relative standard deviation (RSD) ranged from 12.2% to 2.9% for lysozyme concentrations between 0.1 nM and 50μM. These RSD values are reasonable given that there are several steps involved in the procedures. The plot exhibits two regions with different slopes. The lower curve (from 0.1 nM to 5 μM) has a slope (1.41 μA/μM) that is much steeper than that (0.023 μA/μM) of the upper section (from 5 μM to 50μM). And there is a linear relationship between 0.1 nM and 5 μM with a correlation coefficient of 0.9962. The variations in the slopes (sensitivities) between the lower and upper regions may be interpreted on the basis of the surface coverage of the lysozyme molecules confined at the electrode surface. The lysozyme surface density in the lower region is scarce. As a result, the GMBS molecules can readily approach the binding sites of the lysozyme molecules at the surface. The smaller steric hindrance also allows the Fc-SH molecules to position in closer proximity of the underlying Au electrode and the Fc moieties to undergo faster electron transfer reactions. Once the lysozyme concentration exceeds 5 μM, the curve begins to level off, suggesting that most of the PSS adsorbed on the surface had been reacted with lysozyme molecules. Note that a detection level of 0.1 nM of lysozyme can be achieved. Such a low detection level is remarkable considering that no signal amplification scheme is involved in the current method and much lower that those reported by other methods, such as ELISA (35), temperature-independent near-infrared analysis (36), and deep UV laser-induced fluorescence detection (37), etc.

4.2. Voltammetric behavior of Fc-SH linked to surface-confined MT

We also explored the possibility of determination of negatively charged surface-confined proteins based on the above method. Curve a in Figure 5 shows the CV acquired at electrodes covered with MUA and then PAH after electrostatic adsorption of MT, followed by other subsequent surface modifications. The successful immobilization and tagging of MT are evidenced by the

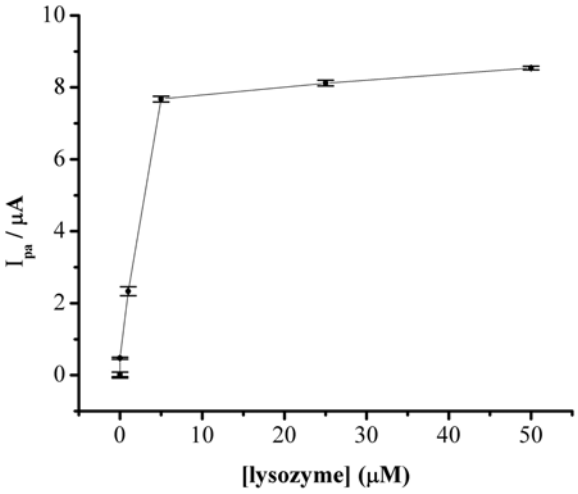

Figure 4. The dependence of anodic peak currents (I_{pa}) on the concentrations of lysozyme. Concentrations of lysozyme determined are 0.1 nM, 1.0 nM, 10 nM, 1.0 μM, 5.0 μM, 25 μM and 50 μM. Other experimental conditions are the same as those in Figure 2. Each point was averaged from at least three replicates and the relative standard deviations are shown as the error bars.

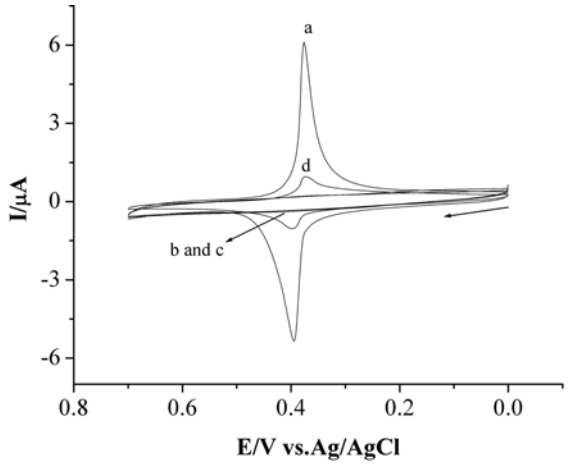

Figure 5. CVs acquired at electrodes covered with MUA and polyelectrolyte in the case of PAH (a) or PSS (b) after electrostatic adsorption of MT from PBS solution containing 100 μM MT, followed by derivatization with GMBS, and the subsequent Fc-SH attachment. The electrochemical response acquired at electrodes covered with PAH in the absence of MT, followed by other subsequent surface modifications is shown as curve c, and curve d was obtained in the absence of PAH. Other experimental conditions are the same as those in Figure 2.

well-resolved voltammetric peaks. The anodic peak appears at 0.395 V with the peak potential separation (ΔE_p) of 0.021 V. For comparison, we performed a control experiment in which PAH was replaced by PSS (curve b). The current in curve b dropped to the background level, indicating that nonspecific adsorption of MT was negligible. Similar to the case of lysozyme detection, no voltammetric peak was acquired at an electrode modified with MUA and then PAH but without having exposed the electrode to the MT solution (curve c). In the absence of PAH, nonspecific adsorption of Fc tags was observed at an electrode modified with MUA after adsorption of MT, followed by other subsequent surface modifications (curve d), further demonstrating the importance of polyelectrolytes in reducing the background signals (26). The above results demonstrated the feasibility of the method to the determination of negatively charged proteins pre-immobilized onto electrode surfaces.

Previously, we have used *N*-(2-ethyl-ferrocene)maleimide (Fc-Mi) to tag cysteine residues on oligopeptides (e.g., glutathione) and obtained relatively reversible voltammograms (38). However, for the case of MT detection, tagging MT pre-immobilized onto electrode surfaces with Fc-Mi results in smaller voltammetric response (Figure 6) in comparison with that in Figure 5a. Such a drastic difference can be ascribed to the number of Fc tags per MT molecule. The thiol-specific electroactive cross-linker Fc-Mi has been used to tag the small number of free cysteine thiols on the MT surface, while in the case of Figure 5a, large quantities of primary amine groups in MT have been derivatized with GMBS and then Fc-SH. It is also worthy to note that facile electron transfer reactions of the Fc tags with the underlying Au electrode occur in Figure 5a. Based on the above results, it appears that a higher labeling efficiency of proteins was achieved using this method.

5. CONCLUSIONS AND PERSPECTIVES

In this work, sensitive and selective method for the determination of egg white lysozyme molecules at ultratrace levels was developed. Lysozyme was first adsorbed onto the PSS-covered electrode via electrostatic interaction. Via the use of the bifunctional reagent GMBS and the redox marker Fc-SH, voltammetric determination of surface-confined redox-inactive lysozyme molecules could be realized. Well-defined ferrocene voltammetric peaks, whose currents increase with the lysozyme concentration, were observed. Analytical figures of merit (e.g. dynamic range, sensitivity, and detection level) were evaluated through the analysis of lysozyme molecules bound to the electrode surface. Concentration levels as low as 0.1 nM can be determined. In addition to the positively charged protein, negatively charged MT can also be detected based on the similar method. The incorporation of polyelectrolytes to electrostatically bind the proteins could eliminate the nonspecific adsorption of the proteins, thus significantly reducing the background signals. Tagging MT pre-immobilized onto electrode surfaces with Fc-Mi results in smaller voltammetric response and slower electron transfer reaction with the underlying electrode. The method is simple, robust, and

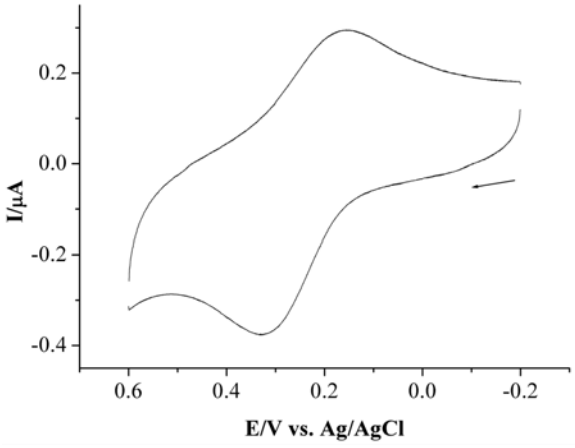

Figure 6. CV acquired at an electrode covered with MUA and then PAH after electrostatic adsorption of MT from PBS solution comprising 100 μM MT, followed by immersing the electrode in a DMSO solution containing 4.0 mM Fc-Mi. The scan rate was 0.05 V s^{-1} and the arrow indicates the scan direction.

selective and holds promise for assaying other proteins at sensitive levels.

6. ACKNOWLEDGEMENTS

Drs Yuan Yuan, Xu Zhu contributed equally to this work. Partial support of this work by the National Natural Science Foundation of China (No. 20775093), and the Shenghua Scholar Foundation of Central South University is gratefully acknowledged.

7. REFERENCES

1. J. Gao, G. J. Opiteck, M. S. Friedrichs, A. R. Dongre & S. A. Hefta: Changes in the protein expression of yeast as a function of carbon source. *J. Proteome Res.* 2, 643-649 (2003)
2. J. Park, S. Kurosawa, J. Watanabe & K. Ishihara: Evaluation of 2-methacryloyloxyethyl phosphorylcholine polymeric nanoparticle for immunoassay of C-reactive protein detection. *Anal. Chem.* 76, 2649-2655 (2004)
3. J. Kovacs-Nolan, M. Phillips & Y. Mine: Advances in the value of eggs and egg components for human health. *J. Agric. Food Chem.* 53, 8421-8431 (2005)
4. O. P. Mishra, S. Yusuf, Z. Ali & G. Nath: Lysozyme levels for the diagnosis of tuberculous effusions in children. *J. Trop. Pediatr.* 46, 296-300 (2000)
5. J. H. R. Kägi & A. Schaeffer: Biochemistry of metallothionein. *Biochemistry* 27, 8509-8515 (1988)
6. E. H. Fischer & E. W. Davie: Recent excitement regarding metallothionein. *Proc. Natl. Acad. Sci. USA* 95, 3333-3334 (1998)
7. D. H. Hamer: Metallothionein. *Annu. Rev. Biochem.* 55, 913-951 (1986)
8. J. Chan, Z. Huang, M. E. Merrifield, M. T. Salgado & M. J. Stillman: Studies of metal binding reactions in metallothioneins by spectroscopic, molecular biology, and molecular modeling techniques. *Coord. Chem. Rev.* 233-234, 319-339 (2002)
9. S.-J. Yi, J. S. Yuk, S.-H. Jung, G. K. Zhavnerko, Y.-M. Kim & K.-S. Ha: Investigation of selective protein immobilization on charged protein array by wavelength interrogation-based SPR sensor. *Mol. Cells* 15, 333-340 (2003)
10. S. E. Mansoor, H. S. Mchaourab & D. L. Farrens: Mapping proximity within proteins using fluorescence spectroscopy. A study of T4 lysozyme showing that tryptophan residues quench bimane fluorescence. *Biochemistry* 41, 2475-2484 (2002)
11. J. Hu, R. S. Sheng, Z. S. Xu & Y. Zeng: Surface enhanced raman spectroscopy of lysozyme. *Spectrochim. Acta, Part A* 51A, 1087-1096 (1995)
12. M. Mueller, B. Kessler, N. Houbenov, K. Bohata, Z. Pientka & E. Brynda: pH dependence and protein selectivity of poly(ethyleneimine)/poly(acrylic acid) multilayers studied by in situ ATR-FTIR spectroscopy. *Biomacromolecules* 7, 1285-1294 (2006)
13. M. Dabrio, A. R. Rodríguez, G. Bordin, M. J. Bebianno, M. D. Ley, I. Šestáková, M. Vašák & M. Nordberg: Recent developments in quantification methods for metallothionein. *J. Inorg. Biochem.* 88, 123-134 (2002)
14. K. Poleć, M. Peréz-Calvo, O. García-Arribas, J. Szpunar, B. Ribas-Ozonas & R. Lobiński: Investigation of metal complexes with metallothionein in rat tissues by hyphenated techniques. *J. Inorg. Biochem.* 88, 197-206 (2002)
15. J. Ellenberg, J. Lippincott-Schwartz & J. F. Presley: Two color green fluorescent protein time-lapse imaging. *Biotechniques* 25, 838-946 (1998)
16. M. Wachsmuth, W. Waldeck & J. Langowski: Anomalous diffusion of fluorescent probes inside living cell nuclei investigated by spatially-resolved fluorescence correlation spectroscopy. *J. Mol. Biol.* 298, 677-689 (2000)
17. A. J. Bard & L. R. Faulkner: Electrochemical methods. Fundamentals and applications. John Wiley & Sons, New York (2001)
18. E. Lojou & P. Bianco: Assemblies of dendrimers and proteins on carbon and gold electrodes. *Bioelectrochemistry* 69, 237-247 (2006)
19. H. Ju & D. Leech: Electrochemical study of a metallothionein modified gold disk electrode and its action on Hg^{2+} cations. *J. Electroanal. Chem.* 484, 150-156 (2000)
20. F. Lisdat & I. Karube: Copper proteins immobilised on gold electrodes for (bio)analytical studies. *Biosens. Bioelectron.* 17, 1051-1057 (2002)
21. K. D. Gleria, C. M. Halliwell, C. Jacob & H. A. O. Hill: Site-specific introduction of an electroactive label into a non-electroactive enzyme (β-lactamase I). *FEBS Lett.* 400, 155-157 (1997)
22. K. D. Gleria, H. A. O. Hill & L. L. Wong: N-(2-ferrocene-ethyl)maleimide: a new electroactive sulphydryl-specific reagent for cysteine-containing peptides and proteins. *FEBS Lett.* 390, 142-144 (1996)
23. H. B. Kraatz: Ferrocene-conjugates of amino acids, peptides and nucleic acids. *J. Inorg. Organomet. Polym.* 15, 83-106 (2005)
24. H. Xie & Z. Q. Gao: Electrochemical activation of glucose oxidase with a 140-fold enhancement in intramolecular electron transfer rate constant. *Front. Biosci.* 10, 2770-2775 (2005)

25. E. Bakker: Electrochemical sensors. *Anal. Chem.* 76, 3285-3298 (2004)
26. T. M. H. Lee, L.-L. Li & I.-M. Hsing: Enhanced electrochemical detection of DNA hybridization based on electrode-surface modification. *Langmuir* 19, 4338-4343 (2003)
27. M. D. Partis, D. G. Griffiths, G. C. Roberts & R. B. Beechey: Cross-linking of protein by ω-maleimido alkanoyl N-hydroxysuccinimido esters. *J. Protein Chem.* 2, 263-277 (1983)
28. J.-W. Jung, S.-H. Jung, H.-S. Kim, J. S. Yuk, J.-B. Park, Y.-M. Kim, J.-A. Han, P.-H. Kim & K.-S. Ha: High-throughput analysis of GST-fusion protein expression and activity-dependent protein interactions on GST-fusion protein arrays with a spectral surface plasmon resonance biosensor. *Proteomics* 6, 1110-1120 (2006)
29. T. X. Zhang, H. Z. Liu & J. Y. Chen: Investigation of affinity interaction between protein and triazine dye in reversed micelles with absorption spectra. *Colloids Surf., A* 196, 79-85 (2002)
30. Z. G. Peng, K. Hidajat & M. S. Uddin: Adsorption and desorption of lysozyme on nano-sized magnetic particles and its conformational changes. *Colloids Surf., B* 35, 169-174 (2004)
31. J. L. Hare & E. Lee: Michael electrophilic addition of biotin to sulfhydryl groups. *Biochemistry* 28, 574-580 (1998)
32. E. A. Smith, M. J. Wanat, Y. F. Cheng, S. V. P. Barreira, A. G. Frutos & R. M. Corn: Formation, spectroscopic characterization, and application of sulfhydryl-terminated alkanethiol monolayers for the chemical attachment of DNA onto gold surfaces. *Langmuir* 17, 2502-2507 (2001)
33. M. Nordberg, G. F. Nordberg & M. Piscator: Isolation and characterization of a hepatic metallothionein from mice. *Environ. Physiol. Biochem.* 5, 396-403 (1975)
34. A. J. Baca, F. M. Zhou, J. Wang, J. B. Hu, J. H. Li, J. X. Wang & Z. S. Chikneyan: Attachment of ferrocene-capped gold nanoparticle/streptavidin conjugates onto electrode surfaces covered with biotinylated biomolecules for enhanced voltammetric analysis. *Electroanalysis* 16, 73-80 (2004)
35. M.-L. Vidal, J. Gautron & Y. Nys: Development of an ELISA for quantifying lysozyme in hen egg white. *J. Agric. Food Chem.* 53, 2379-2385 (2005)
36. S.-Y. B. Hu & M. A. Arnold: Temperature-independent near-infrared analysis of lysozyme aqueous solutions. *Anal. Chem.* 72, 696-702 (2000)
37. P. Schulze, M. Ludwig, F. Kohler & D. Belder: Deep UV laser-induced fluorescence detection of unlabeled drugs and proteins in microchip electrophoresis. *Anal. Chem.* 77, 1325-1329 (2005)
38. Y. Wang, X. Zhu, Z. Hu, J. Wang & F. Zhou: Voltammetric determination of surface-confined biomolecules with N-(2-ethyl-ferrocene)maleimide. *Electroanalysis* 17, 2163-2169 (2005)

Abbreviations: GMBS: 4-maleimidobutyric acid N-hydroxysuccinimide ester, Fc-SH: 11-ferrocenyl-1-undecanethiol, PSS: poly (sodium 4-styrenesulfonate), PAH: poly(allylamine hydrochloride), MT: metallothionein, ELISA: enzyme-linked immunosorbent assay, RIA: radioimmunoassay, MUA: 11-mercaptoundecanoic acid, Fc-Mi: *N*-(2-ethyl-ferrocene)maleimide, PBS: phosphate buffered saline, CV: cyclic voltammogram, ΔE_p: peak potential separation, I_{pa}: anodic peak current, RSD: percent relative standard deviation.

Key Words: Surface-confined, Lysozyme, MT, Voltammetry

Send correspondence to: Dr. Jianxiu Wang, College of Chemistry and Chemical Engineering, Central South University, Changsha, Hunan, P. R. China 410083, Tel: 86731836954, Fax: 867318836964, E-mail: jxiuwang@mail.csu.edu.cn

http://www.bioscience.org/current/vol13.htm