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An electrochemical biosensor for analysis of Fenton-mediated oxidative damage to BSA using poly-*o*-phenylenediamine as electroactive probe

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ABSTRACT

A sensitive electrochemical procedure based on bovine serum albumin (BSA)/poly-*o*-phenylenediamine (PoPD)/carbon-coated nickel (C-Ni) nanobiocomposite film modified glassy carbon electrode (BSA/PoPD/C-Ni/GCE) has been developed to explore the electrochemical detection of BSA damage induced by hydroxyl radical. It is the first time that the electrochemical method has been applied for the analysis of Fenton-mediated oxidative damage to proteins. The hydroxyl radical was generated by Fenton reaction (Fe^{2+}/H_2O_2), which was also validated by ultraviolet–visible (UV–vis) spectroscopy. The decrease in intensity of the PoPD oxidation signals was used as an indicator for the detection of BSA damage. Damage to BSA was also validated by horizontal Attenuation Total Reflection Fourier Transform Infra-red (ATR-FTIR) spectroscopy and the change of protein carbonyl group content achieved by UV–vis spectroscopy. Effects of H_2O_2 concentration, the ratio of Fe^{2+} and H_2O_2 and incubation time on BSA damage were examined. Protections of BSA from damage by antioxidants were also investigated. These conclusions demonstrated that the proposed electrochemical method is expected to the further application for protein damage studies.

1. Introduction

Damage focused on DNA, protein mediated by reactive oxygen species (ROS) was widely reported (Jeffrey et al., 1999; Tatsiana et al., 2006; Shacter, 2000). ROS, principally free radicals, such as superoxide anion, hydrogen peroxide, and hydroxyl radical, are highly unstable molecules with available electrons, generated in vivo during metabolic processes (Jeffrey et al., 1999). Biomolecules, including proteins, enzymes, lipids, carbohydrates, DNA and nearly all tissues in humans and animals (Tatsiana et al., 2006; Shacter, 2000) were easy to be attacked by ROS. ROS were implicated in a variety of health problems such as aging, cardiovascular disease, AIDS, and cancer.

Free radicals-mediated damage to DNA has received scientists' attention since DNA's key role in life processes. Numerous studies have reported the DNA damage caused by ROS, both in vivo and in vitro (Tang et al., 1998; Daniels et al., 1998; Jean et al., 2010). Concretely, free radicals interacts with DNA may induce DNA strand breaks, oxidative DNA base modifications (Aiyar et al., 1991; Anderson and Collery, 1990). One of the most abundant and easily measured products of oxidation is 8-hydroxydeoxyguanosine (8-OH dG), which is a key biomarker relevant to carcinogenesis (Cheng et al., 1996). If damaged DNA goes unrepaired, it can mutate and eventually lead to cancer (Weinberg, 1989; Susanne et al., 2001).

Recently, protein damage induced by free radicals was also received scientists' attention. Proteins are most likely to be attacked as a result of their abundance in cells (proteins compose ca. 70% of the drymass of most cells). In particular, protein oxidation seems to be important in several neurodegenerative and neuroinflammatory diseases (Gerhard, 2006; Stadtman, 1992). These diseases were relevant to the accumulation of oxidized proteins within neurons or extracellular tissues. Oxidative modified proteins may undergo oxidation of residues on protein side chains, protein fragmentation, protein–protein cross-linking, DNA–protein cross-linking, function of enzymes losing, conformational change, charge properties change, attenuation of conductibility. An electrochemical biosensor for detection of tyrosine oxidation induced by hydroxyl radicals were reported (Qu et al., 2011). Almost all amino acids in proteins are sensitive to free radicals, especially amino acids containing sulfydryl groups, such as cysteine, methionine, tyrosine, and tryptophan. The increase of carbonyl groups has been widely used as a marker of ROS-mediated protein damage. Levine et al. (1990) showed free radical damage to protein by measuring

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carbonyl groups content. Davies et al. (1987) showed the direct evidence of amino acid in bovine serum albumin (BSA) modification after exposure to free radicals.

A great deal of studies have reported biomacromolecule damage induced by free radicals (Susanne et al., 2001; Salvi et al., 2001), and antioxidant's efficacy in reducing damage (Teixeira et al., 2005). Determination of oxidative damage in humans is very useful in order to study the effects of free radicals on human health and quality inhibition effect of antioxidant enzymes and small molecule antioxidants. It will provide theoretical basis on disease diagnosing, anti-aging anti-cancer drug screening and drug analyzing. Presumptively, the investigation will provide useful information on protecting protein from oxidative damage. Comet assay, which is single cell gel electrophoresis, was widely used to explore DNA damage (Martin et al., 2003; Gilbert et al., 2005). The process yield a pattern suggestive of the appearance of a comet, for which the tail length relates to the damage (Martin et al., 2003). Common approaches to detect protein damage were electrophoresis, radioimmunoassay, high performance liquid chromatography (HPLC) and mass spectroscopy (MS) (Tan et al., 1999; Aoife et al., 1999; Skinner and Malpaux, 1999), and carbonyl groups content measuring. Few workers have focused on the electrochemical method in protein damage. Electrochemical sensors (Mugwerua and Rusling, 2006; Wang et al., 2010) have attracted much attention because of its quick response, relatively high sensitivity, the ability to be miniaturized, and have been successfully applied for monitoring DNA damage (Wang et al., 2009).

In this paper, protein damage was, for the first time, investigated by electrochemical method based on the attenuation of conductibility after damage caused by free radical. Oxidation signal of poly-*o*-phenylenediamine (PoPD) was used as an indicator for the sensitive detection of BSA damage based on evidently attenuation of conductibility. The attenuation of conductibility in BSA was also confirmed by electrochemical impedance spectroscopy (EIS). Radicals-induced BSA damage was validated by protein carbonyl group content measurement and changes in protein conformation.

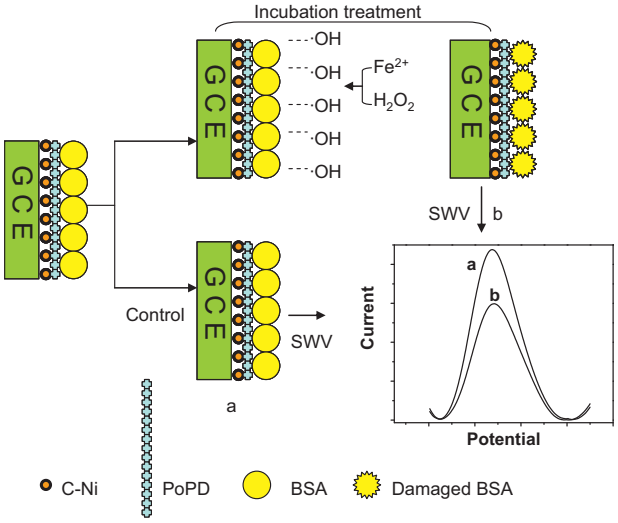
2. Experimental

2.1. Chemicals and reagents

BSA (Tianyuan) was purchased from Sigma (St. Louis, MO, USA) and accepted for use without further purification. The standard stock solutions of 0.68 mg/mL BSA were prepared by directly dissolving BSA in double-distilled water. C-Ni nanoparticles (the average diameter 10–50 nm using methods described by Ling et al., 2003) were obtained from Professor Xungao Zhang (the Center for Analysis and Measurement, Wuhan University, Wuhan, China). *O*-phenylenediamine (oPD) (Ardrich Chemical Co.) was sublimated under vacuum at 80 °C before use. 2,4-Dinitrophenylhydrazine (DNPH) (10 mM) was prepared by 2 M HCl. Other chemicals were of analytical-regent grade. All solutions were prepared with double-distilled water.

2.2. Apparatus

Electrochemical measurements were performed by a model CHI660C electrochemical workstation (CH Instruments, Chenhua Co., Shanghai, China). A three-electrode system was used in the measurements, with a bare GCE (3 mm in diameter) or nanobiocomposite film modified GCE as the working electrode, a saturated calomel electrode (SCE) as the reference electrode and a platinum wire as the auxiliary electrode. All the following potentials reported in this work were against the SCE. EIS measurements were performed in the presence 5 mmol/L $[Fe(CN)_6]^{3-/4-}$ solution containing 0.1 mol/L KCl. Ultraviolet–visible (UV–vis) spectroscopy was carried out with UV 2300 spectrophotometer (Shanghai Tian Mei Scientific Instrument Co., Ltd., China). The infra-red (IR) spectroscopy was achieved by horizontal Attenuation Total Reflection Fourier Transform IR (ATR-FTIR) Spectrometer (spectrum One, Perkin Elmer, USA).



Scheme 1. Schematic diagram of BSA damage.

2.3. Procedure

2.3.1. Construction of BSA/PoPD/C-Ni/GCE

Fabrication of BSA/PoPD/C-Ni/GCE was in accordance with our previously published methods (Feng et al., 2011). Generally speaking, 10 μL of 1.0 mg/mL C-Ni nanoparticles suspension (1.0 mg C-Ni nanoparticles in 1.0 mL sodium dodecyl sulfate (SDS) (5.0 mmol/L)) was dropped onto the surface of a clean GCE, followed by evaporating the solvent under an infrared lamp. PoPD/C-Ni/GCE was formed by dipping C-Ni/GCE into 5.0×10^{-4} mol/L of oPD solution prepared using 0.1 mol/L H_2SO_4 solution and then scanning in the potential range from −0.5 to +1.0 V at 100 mV/s for 6 cycles. After being rinsed with phosphate buffer solution (PBS) solution (pH 5.0), the PoPD/C-Ni/GCE coated with BSA solution for 30 min to immobilize the BSA on the modified surface through electrostatic force, followed by washing the electrode with 0.2% SDS solution and then rinsing it with purified water to remove the un-immobilized BSA. The resulting BSA/PoPD/C-Ni/GCE was used for the electrochemical measurements.

2.3.2. Incubation of sensors with Fenton's reagent and antioxidant

Scheme 1 shows the detection method of BSA damage induced by hydroxyl radicals. Sensor response was obtained by square wave voltammetry (SWV) before incubation. Then the modified electrodes were incubated in 4 mL pH 5.0 PBS containing 12.5 mM $FeSO_4$ and 50 mM H_2O_2 (the cleavage agent), with stirring, followed by washing and subsequent SWV analysis. During the course of the incubation treatment in the cleavage agent, the iron ion and H_2O_2 reacted to produce •OH (Zhao et al., 2005), •OH attacked the BSA in the film and induced BSA damage. SWV peak current difference of the probe PoPD after (Scheme 1b) and before (Scheme 1a) incubation in the cleavage agent was used as the measurement signal for BSA damage. SWVs were at 4 mV step, 25 mV pulse, and 15 Hz in 0.1 M PBS buffer pH 7.0. Considering the variation for replicative electrode, the relative peak current ratio (i/i_0) (Liu and Hu, 2008) instead of absolute peak current was used to evaluate the effect of

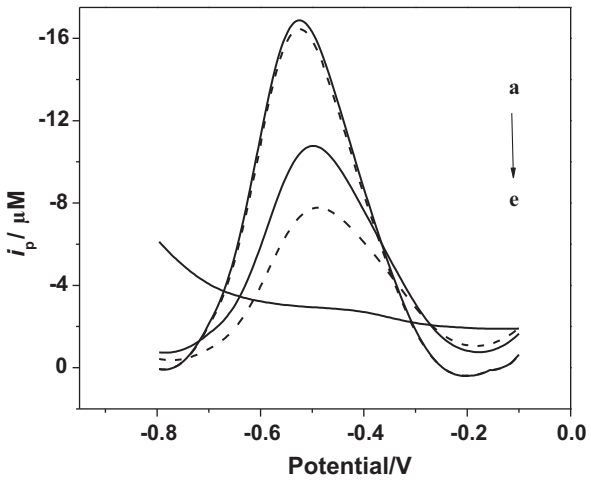


Fig. 1. SWVs in 0.1 M PBS (pH 7.0) of PoPD/C-Ni/GCE (a and b), BSA/PoPD/C-Ni/GCE (c and d), C-Ni/GCE (e) before (solid line) and after (dashed line) incubation with 12.5 mM $FeSO_4$ and 50 mM H_2O_2 for 30 min (scan rate of 0.1 V s^{-1}).

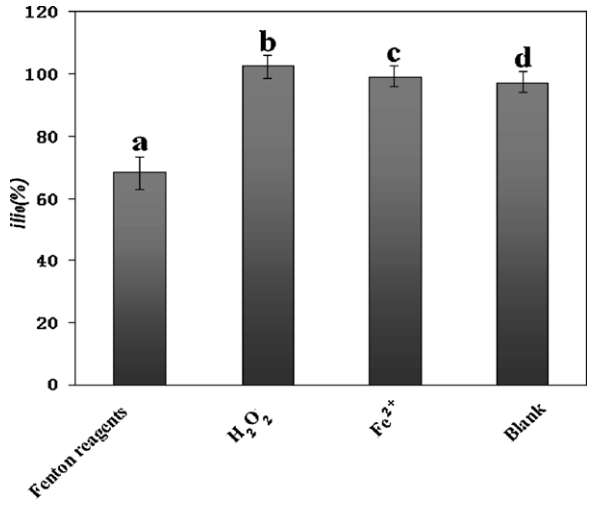


Fig. 2. SWV oxidative peak current ratio i/i^0 of BSA/PoPD/C-Ni/GCE before and after incubation for 30 min with (a) Fe^{2+}/H_2O_2 = 12.5/50 mM; (b) 50 mMH_2O_2; (c) 12.5 mM Fe^{2+}; (d) blank.

BSA damage, where i_0 and i represent the SWV oxidation peak current for the films before and after incubated in the Fenton reagents, respectively.

Dependence of damage degree on the molar ratio of Fe^{2+}/H_2O_2, H_2O_2 concentration, and incubation time are described in Supplementary Figs. S1, S2 and S3, respectively.

3. Results and discussion

3.1. Verification of hydroxyl free radicals in Fenton system

Electron spin resonance (ESR) (Pou et al., 1994; Stokes et al., 1994), HPLC (Zielonka et al., 2009), and spectrophotometry (Grootveld and Rhodes, 1995) were used for the determination of •OH. Both ESR and HPLC required expensive equipments and complex operations. In contrast, spectrophotometry method is a simple approach for general laboratory. Methyl violet (MV) was introduced to measure hydroxyl radicals with UV–vis spectroscopy in present work. The difference in absorption bands of MV appearing at about 580 nm was employed to confirm generation of hydroxyl free radicals (Fig. S4). In MV, the carbon–carbon double bonds with high electron cloud density was easy to be attacked by hydroxyl free radicals. Thereby electrophilic addition reaction took place, leaving MV fade. The basic principle is shown in previously published methods (Grootveld and Rhodes, 1995). The results suggested that free radicals generated in the experimental condition.

3.2. Electrochemical behaviors of BSA/PoPD/C-Ni/GCE

Voltammetric sensors featuring nanobiocomposite film containing C-Ni, BSA, PoPD were evaluated to monitor BSA damage reactions. SWVs of PoPD/C-Ni/GCE electrode (in the absence of BSA) before and after incubation with 12.5 mM $FeSO_4$ and 50 mM H_2O_2 for 30 min have been presented in Fig. 1. As can be seen from the figure (curves a and b), in the absence of BSA, PoPD oxidation signals kept almost unchanged after incubation with Fenton reagent. However, in the presence of BSA, obvious current decrease after incubation in the cleavage reagent was observed (curves c and d), which may be due to evidently attenuation of BSA conductibility induced by Fenton reagents (Davies et al., 1987). The more detailed mechanism needs further research.

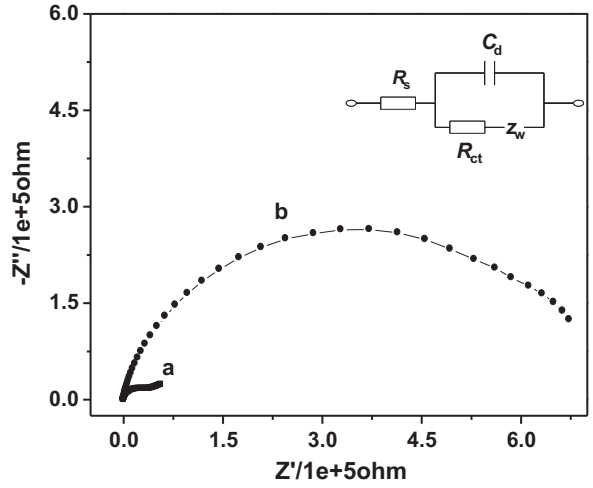


Fig. 3. EIS spectra of the BSA/PoPD/C-Ni/GCE in 0.1 M $K_3Fe(CN)_6/K_4Fe(CN)_6$ (0.1 M KCl) before (a) and after (b) incubation with 12.5 mM $FeSO_4$ and 50 mM H_2O_2 for 30 min. Inset: Randle's equivalent circuit used to model impedance data in the presence of redox couples.

3.3. Scheme of the BSA damage

The peak current decreased apparently in the presence of Fe^{2+} and H_2O_2 (Fig. 2a). As a contrast, incubation in H_2O_2 (Fig. 2b), Fe^{2+} (Fig. 2c) and blank (Fig. 2d) separately have also been done. No evident signal variation was observed in the control solutions. From the above control experiments, it can be concluded that BSA damage was caused by hydroxyl radical and the proposed method can be used for the investigation of BSA damage expediently.

It is well known that EIS is a powerful tool for investigating the interface properties of chemically modified electrode surface and affording information on the impedance changes of the electrode surface during the modification process (Huang et al., 2010). Fig. 3 shows the Nyquist plots of different electrodes in the presence of 5 mmol/L $[Fe(CN)_6]^{3-/4-}$ solution, which were fitted by digital simulation based on the equivalent circuit (Fig. 3, inset). The electron transfer process is caused by the presence of $[Fe(CN)_6]^{3-/4-}$ couple in the bulk solution. The EIS includes a semicircular part and a linear part. The semicircular part at higher frequencies corresponds to the electron-transfer limited process and the diameter is

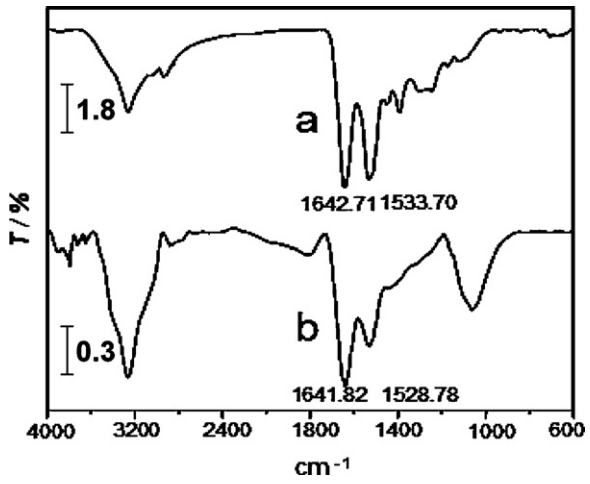


Fig. 4. ATR-FTIR spectrum for before (a) and after (b) incubation with 12.5 mM $FeSO_4$ and 50 mM H_2O_2 for 30 min.

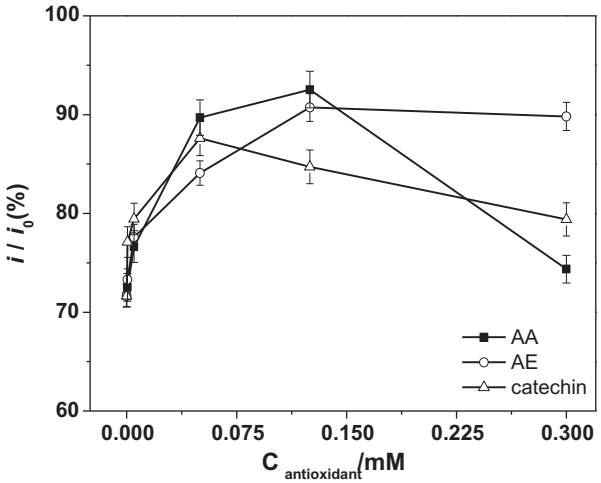


Fig. 5. Influence of concentrations of different antioxidant on the ratio of final to initial SWV peak current for sensors with BSA/PoPD/C-Ni films. The following symbols denote treatment with Fenton reagent for 30 min AA (■), AE (○), and catechin (△).

equivalent to the charge-transfer resistance (Rct). For the modified electrode after incubation with Fenton reagent, the Rct increased from 40 000 Ω (Fig. 3a) to 750 000 Ω (Fig. 3b), which is reflected by the appearance increase in the semicircular part of the spectrum, indicating that damaged BSA hinder the charge transfer. Apparently decrease in peak current after damage may be due to evidently attenuation of BSA film conductibility induced by hydroxyl radicals. This inference was validated by EIS measurements.

3.4. Validation of BSA damage in Fenton system

Exposure of proteins to $^{\bullet}OH$, $O_2^{\bullet -}$ or both leads to gross structural modifications. Protein carbonyl groups were usually used as biomarkers of oxidative stress (Levine et al., 1990). In our present work, the apparent change appeared simultaneously in IR and UV illustrated that BSA damage mediated by the Fenton reaction involves the increasing in carbonyl content of proteins. The carbonyl content of proteins was quantified by the reaction with DNPH, using the method described by Levine et al. (1990). UV–vis spectroscopy was shown in Supplementary Fig. S5.

ATR-FTIR spectral band shapes can be used to monitor changes in protein conformation in present work (Nassar et al., 1995; Susi and Byler, 1986). The shapes of amide I (1700–1600 cm^{-1}) and amide II (1600–1500 cm^{-1}) infrared absorption bands of proteins provide detailed information on the secondary structure of the polypeptide chain (Spiro and Strekas, 1974). Amide I was attributed to stretching vibration of C=O on the polypeptide chain of protein backbone, while amide II was due to bending vibration of N–H bond and stretching vibration of C–N. Control experiments were incubated in absence of Fenton reagents and hence in absence of hydroxyl radicals showed bigger transmittance (Fig. 4a). BSA that had been denatured with Fenton Reagents and then cast as films had smaller transmittance (Fig. 4b). Its amide I and amide II transmittance weaken obviously (Spiro and Strekas, 1974). To highlight the relative infrared transmittance of Amide I and amide II, different legends were adopted for curves a and b. From the relative infrared transmittance of amide I and amide II, it can be speculated that amide I which was related to carbonyl on the polypeptide chain of protein backbone increased comparatively. Therefore, ATR-FTIR validated the carbonyl content of proteins enhanced after damage. This conclusion was consistent with foregoing deduction obtained from UV absorption experiment.

3.5. Protection of BSA from damage by antioxidants

Several small molecule antioxidants including ascorbic acid (AA) (Mugwerua and Rusling, 2006), aloe-emodin (AE) (Arosio et al., 2000), catechin (Wang et al., 2008) and others (Frei, 1999) were reported to be excellent free radical scavengers. Fig. 5 shows the influence of the antioxidant concentration on the ratios of peak current of sensors after and before incubation in the cleavage agent. The increases in the relative ratio of peak current implied an effective protection effect of antioxidants. Control experiments aimed at investigating whether antioxidant were excellent free radical scavengers, the modified electrode was incubated in Fenton reagent without antioxidant for 30 min, corresponding to the data as concentration of antioxidant was zero. AA and catechin showed the least signal decrease at concentration of 0.125 mM and 0.04 mM, respectively. The curve for AE leveled off up to about 0.125 mM. Conclusively, AA, AE, and catechin are strong antioxidants that are capable of scavenging hydroxyl radicals. But in terms of AA and catechine, the ratio of peak current decreased after the peak value, indicating property of promoting oxidation. The prooxidative capacities of AA and catechin are accordance with paper reported (Samuni et al., 1983; Mochizuki et al., 2002).

3.6. Reproducibility and stability

Stability and fabrication reproducibility were important parameters for modified electrode. For 10 successive determinations at the same modified electrode, the relative standard deviation (R.S.D.) was 2.08%. Fabrication reproducibility was estimated with five different electrodes, which were constructed by the same procedure, R.S.D. was 7.97%. Moreover, under the same condition, SWV current of modified electrode decreased to 87.96% after six days use. As a result, BSA/PoPD/C-Ni/GCE exhibited good reproducibility and comparative stability.

4. Conclusions

Sensitive square wave voltammetric detection of BSA damage induced by hydroxyl radical using PoPD as the electroactive probe was proposed. Electrochemical detection in present paper approved evidently attenuation of BSA conductibility after damage. Dependences of damage degree on the molar ratio of Fe^{2+}/H_2O_2, H_2O_2 concentration, and incubation time were studied using the

proposed method. The fabricated sensor exhibited good stability and reproducibility. Scheme of BSA damage was also speculated. BSA damage and generation of hydroxyl free radicals were validated through UV–vis spectroscopy and ATR-FTIR spectroscopy. Effects of some common antioxidants were also explored. Antioxidants implied an effective protection effect in a certain range of concentration. The present electrochemical method is a significative supplement to investigation of protein damage and expected to further application to BSA damage studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.07.021.

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