An electrochemical biosensor for the detection of tyrosine oxidation induced by Fenton reaction

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ABSTRACT

A simple and sensitive electrochemical biosensor was used to detect tyrosine oxidation induced by hydroxyl radicals generated by Fenton reaction ($\text{Fe}^{2+}/\text{H}_2\text{O}_2$). Poly(glu, tyr) (4:1) peptides were immobilized on indium tin oxide (ITO) electrode surface via layer-by-layer assembly technique, and $\text{Os}(\text{bpy})_3^{2+}$ -mediated tyrosine oxidation current was employed as the signal reporter of the biosensor. It was found that the electrochemical signal of the peptide decreased markedly after incubation with Fenton reagents. Interestingly, L-dopa, the oxidation product of tyrosine, was likely to form complexes with $\text{Fe}(\text{III})$, which could suppress the electro-oxidation of L-dopa and resulted in decrease of current response. Our results indicate that the peptide damage involved two steps and was a second-order reaction. X-ray photoelectron spectroscopy was used to quantitatively determine nitrogen elemental percentage on peptide-coated electrode surface, which eliminated the possibility that signal decrease was caused by peptide backbone cleavage. Moreover, the lowest concentration of Fenton reagents that could be detected was $10 \mu\text{M}$ Fe^{2+} or H_2O_2 , similar to the level in vivo. We suggest that the biosensor can be used to detect protein damage induced by Fenton reaction.

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1. Introduction

It is commonly recognized that oxidative damage to proteins is involved in a number of pathologies including cancers and aging process (Berlett and Stadtman, 1997; Hensley and Floyd, 2002). Reactive oxygen species (ROS) induce a large range of protein oxidation caused by various amino acid modifications, which are involved in hydroxylation of aromatic groups and aliphatic amino acid side chains, and conversion of some amino acid residues to carbonyl derivatives (Stadtman and Levine, 2003). Most of these modifications can be used as useful markers for total protein oxidation.

The interaction of iron ions with hydrogen peroxide (commonly referred to as the Fenton reaction) in biological systems can lead to the formation of hydroxyl radicals, which can directly damage most biomolecules and are thought to be highly reactive tissue-damaging species in vivo (Halliwell and Gutteridge, 1984). Many studies have shown the evidences that Fenton reaction plays important roles in protein carbonylation and lipid peroxidation. However, its significant effect is most likely to be upon DNA. Linn's

group has made a systematic study on the formation, prevention and repair of DNA damage by iron-mediated Fenton reaction (Henle and Linn, 1997). Stadtman and co-workers have demonstrated site-specific nature of metal-catalyzed protein oxidative modifications induced by Fenton reaction, which can destroy catalytic activity or structural function (Stadtman and Oliver, 1991). The detailed chemical mechanisms of various amino acid oxidative damage reactions have been elucidated in their study. They have also investigated the relationships between the accumulation of oxidized protein in tissues of aging animals and a number of age-related diseases (Berlett and Stadtman, 1997).

Tyrosine derivatives such as dityrosine, Dopa have been used as indicators of oxidative stress because tyrosine is present in significant quantities in vivo, and readily reacts with electrophilic free radicals to form stable derivatives. Many studies have detected tyrosine oxidation products in serum, cerebrospinal fluid, saliva, brain and eye lens. Different kinds of tyrosine oxidation products in tissues indicate the relevant mechanism of oxidation. Dityrosine, as one of important biomarkers for oxidative stress of tyrosine formed in the process of protein post-translational modification, can be generated by peroxidase-catalyzed mechanism (Giulivi et al., 2003), or by reaction with the reactive nitrogen species. Dopa is usually used as an index of tyrosine with direct attack of hydroxyl radicals (Cohen et al., 1998). Increases of tyrosine oxidation product level in oxidatively damaged protein have been associated with

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a wide range of diseases such as eye cataracts (Wells-Knecht et al., 1993), atherosclerosis, Parkinson's and Alzheimer's diseases. More interest in tyrosine oxidation products as clinical indicators of oxidative modification has driven the development of important techniques for the specific analysis and quantification of these molecules. There are several classic detection methods for analysis of tyrosine oxidation. For instance, immunoassay is facilitated by some commercially available antibodies against oxidized tyrosine products, but this method is considered to be semi-quantitative (Khan et al., 1998). Mass spectrometry has become an important analytical technique for quantitative measurement of dityrosine (DiMarco and Giulivi, 2007) in recent years, which is usually coupled with GC or HPLC. Unfortunately, the high expense and expertise required as well as extensive sample preparation may make this method less readily accessible for common use. As a powerful and flexible tool for basic and clinical research, HPLC–ECD is one of the main detection methods for tyrosine oxidation because most of the oxidized products are electro-active. It has been demonstrated that tyrosine and its six oxidation products can be determined by HPLC–ECD at the same time (Hensley et al., 1999). Recently, tyrosine-containing fluorescent proteins were employed for detection of oxidative stress with high sensitivity (Espey et al., 2002; Tsourkas et al., 2005).

Due to its low cost, simple operation and quick detection, electrochemistry has become a popular and useful method for detecting protein modification. Kerman's group has reported electrochemical detection of human chorionic gonadotropin hormone (hCG) in urine and total prostate-specific antigen (T-PSA) amyloid peptides related to Alzheimer's disease by monitoring the current signal changes of tyrosine and tryptophan residues in antibody (Kerman et al., 2006; Okuno et al., 2007). Additionally, detection of inhibition on enzyme-catalyzed tyrosine phosphorylation on magnetic beads was also carried out by following the increase of tyrosine oxidation current on screen-printed carbon electrode (Kerman et al., 2007).

In this study, an electrochemical biosensor was employed to detect tyrosine oxidation induced by Fenton reaction. We found that the tyrosine in peptide was oxidized by hydroxyl radicals generated from the Fenton reaction. The process of oxidative damage involved two steps: oxidation of tyrosine to L-dopa, and subsequent formation of Fe(III)–L-dopa complex, which suppressed the electroactivity of tyrosine oxidation product. The established biosensor was able to detect peptide oxidation by Fenton reagents at levels similar to in vivo concentrations. To our knowledge, this is the first time that an electrochemical peptide biosensor has been used to detect tyrosine oxidation induced by Fenton reaction.

2. Experimental

2.1. Reagents and materials

L-tyrosine was obtained from Shanghai Biochemicals (Shanghai, China). Poly-(diallyldimethyl ammonium chloride) (PDAA), and poly(glu, tyr) (4:1) sodium salt were obtained from Sigma Aldrich (St. Louis, MO, USA) and used as received. Desferrioxamine (DFO) was obtained as the mesylate salt (Desferal) from Ciba-Geigy (Basle, Switzerland). Iron sulfate heptahydrate was from Acros (Geel, Belgium). Hydrogen peroxide was from Alfa Aesar (Ward Hill, MA). All other chemicals and solvents were of analytical grade. Indium tin oxide conductive glass was supplied by Weiguang Corp (Shenzhen, P.R. China). The metal complex Os(bpy)₃Cl₂ was prepared according to the published procedures described elsewhere (Hamann et al., 2005). ITO electrodes (working area 25 mm²) were fabricated and cleaned according to the established procedure (Qu et al., 2008).

Scheme 1. Schematic diagram of layer-by-layer assembled peptide sensor surface.

2.2. Fabrication of electrochemical biosensor

Peptides were immobilized on ITO surface by layer-by-layer electrostatic adsorption technique, as depicted in Scheme 1. First, an ITO electrode was immersed in positively charged PDAA solution (2 mg mL^{−1} in 20 mM phosphate, pH 7.4) for 1 h. After rinse with water, the electrode was then immersed for 30 min in a peptide solution in 20 mM phosphate, pH 7.4. Cyclic voltammetry was performed on CHI 660B electrochemistry analyzer (Austin, TX, USA) with a Pt counter electrode and 3 M Ag/AgCl reference electrode, and with 20 mM phosphate buffer pH 7.4 as the supporting electrolyte.

2.3. Peptide damage reaction

Poly(glu, tyr) (4:1) (100 μg/mL) on ITO surface was damaged by reacting with Fe²⁺/H₂O₂ in pure water. The reaction was incubated at 37 °C for the required time, and then the electrode was taken out and rinsed with pure water.

2.4. ESI-Q-TOF-MS characterization of tyrosine oxidation product

Analysis by electrospray ionization quadrupole time-of-flight mass spectrometry of the tyrosine oxidation product was performed on a Micromass Q-TOF mass spectrometer. MS and MS/MS spectra were acquired in positive-ion mode. Capillary and sample cone voltages were 3.5 kV and 20 V; source and desolvation temperatures were 80 °C and 200 °C, respectively. Nitrogen was used as the drying gas. The collision gas was argon at a pressure of 5.0 × 10^{−5} Torr, and the collision energy was 20 V. Both the high and low resolution for mass filter was set at 10.0 V. The range between *m/z* 50 and 500 was recorded at a resolution of 5000 (FWHM), and the accumulation time was 1 s per spectrum. For MS/MS studies, the quadrupole was used to select the parent ions, which were subsequently fragmented in a hexapole collision cell by using argon as the collision gas. Data acquisition and analysis were carried out by using MassLynx software (Waters; Version 4.0).

2.5. X-ray photoelectron spectroscopy (XPS) characterization of N element

XPS analysis was carried out using a ESCALAB 250 (ThermoFisher Scientific USA). The X-ray source was a monochromated Al K α line (1486.6 eV). Analyser mode is CAE. The analyzer was operated with pass energy of 200 eV and high-resolution scans were accumulated with 30 eV.

3. Results and discussion

3.1. Detection of tyrosine oxidation in solution by Fenton reaction

We have detected tyrosine phosphorylation by an electrochemical sensor in our previous study (Qu et al., 2008). The sensor was based on the differential electrochemical reactivity

Fig. 1. (A) Cyclic voltammograms of ITO electrodes in 3 μM $\text{Os}(\text{bpy})_3^{2+}$ (a) 180 μM tyrosine, (b) 180 μM tyrosine and H_2O_2 (0.4 mM), (c) 180 μM tyrosine and FeSO_4 (0.1 mM), (d) 180 μM tyrosine and FeSO_4 (0.1 mM)/ H_2O_2 (0.4 mM), and (e) 3 μM $\text{Os}(\text{bpy})_3^{2+}$. (B) Cyclic voltammograms of ITO electrodes in 3 μM $\text{Os}(\text{bpy})_3^{2+}$ (a) 180 μM L-dopa, (b) 180 μM L-dopa and H_2O_2 (0.4 mM), (c) 180 μM L-dopa and FeSO_4 (0.1 mM), (d) 180 μM L-dopa and FeSO_4 (0.1 mM)/ H_2O_2 (0.4 mM), and (e) 3 μM $\text{Os}(\text{bpy})_3^{2+}$.

between unphosphorylated and phosphorylated tyrosines. Using osmium tris(bipyridine) as an electrocatalyst, the electro-oxidation response of tyrosine was turned off when tyrosine became phosphorylated. In this study, we aimed to develop an electrochemical sensor to detect tyrosine and protein oxidation induced by Fenton reagents. We hypothesized that, when tyrosine gets oxidized, its phenol group (responsible for tyrosine electrochemical reaction) would be transformed into something electrochemically inactive, thus leading to loss of electrochemical signal. As shown in Fig. 1(A), the anodic current of tyrosine amino acid dissolved in an electrolyte solution was very high in the presence of $\text{Os}(\text{bpy})_3^{2+}$, but decreased remarkably after addition of the Fenton reagents $\text{Fe}^{2+}/\text{H}_2\text{O}_2$. However, addition of either H_2O_2 or Fe^{2+} alone has no significant effect on the current response. The results demonstrated that tyrosine was oxidized by the hydroxyl radicals generated from the Fenton reaction and changed its electrochemical behavior. An interesting question was raised: what is the product of tyrosine oxidation that caused the electrochemical current to decrease? ESI-Q-TOF-MS was employed in order to identify the oxidation product of tyrosine. As shown in Fig. S1, there is a new peak formed at m/z 198 in the mass spectrum of tyrosine with the Fenton reagents, suggesting L-dopa might be formed, which was further supported by MS-MS analysis (shown in Fig. S2). The result is consistent with published result (Cohen et al., 1998).

3.2. Electrochemical behavior of L-dopa under Fenton reaction

Previous studies have proved that L-dopa shows reversible electro-activity at chemically modified carbon electrode (Sivanesan and John, 2007). In our experiments, we found that L-dopa was not as stable as tyrosine because the catechol group could be readily oxidized to dopaquinone, which undergoes subsequent cyclization to produce cyclodopa in solution. Fig. 1(B) shows the electrochemical behavior of L-dopa in PB containing 3 μM $\text{Os}(\text{bpy})_3^{2+}$. L-dopa shows good electro-activity on ITO surface, with even higher current response than tyrosine at the same concentration. Then the question is why the current signal of tyrosine decreased after oxidation to L-dopa? It is clear that the current response of L-dopa decreased obviously after reacting with Fenton reagents. Fe^{2+} could be oxidized to Fe^{3+} in Fenton reaction and tyrosine to L-dopa subsequently. Therefore a possible reason causing current signal decrease after reaction with Fenton reagents might be due to Fe^{3+} chelation by the catechol group of L-dopa. We suggest that the formation of $\text{Fe}(\text{III})$ -Dopa complex may inactivate the electrochemical behavior of catechol group of L-dopa, which resulted in the decrease of current response for tyrosine after reaction with

Fenton reagents in our study. In addition, Fe^{3+} may also oxidize catechol group to its corresponding quinone form, therefore, preventing the electrochemical oxidation of these groups. Sever et al. have found that $\text{Fe}(\text{Dopa})_3$ cross-link is the key for the generation of marine-mussel adhesive. Their studies with the dopa-containing extracted protein and peptide models provided evidence for the presence of $\text{Fe}(\text{Dopa})_3$ in this common biological material (Sever et al., 2004). Inspired by the composition of adhesive proteins in mussels, Lee et al. developed a facile method for surface modification in which self-polymerization of dopamine formed an adherent polydopamine coating onto a wide variety of inorganic and organic materials (Lee et al., 2007). As shown in Fig. 1(B), the current signal of shoulder peak at 0.4 V in the presence of $\text{Fe}(\text{II})$ alone is much higher than in other situations and the current response at 0.7 V decreased accordingly. The possible reason might be that formation of complex between $\text{Fe}(\text{II})$ and L-dopa changed the electrochemical behavior of L-dopa. The results are consistent with a previous study (Brooksby et al., 2008), in which Brooksby studied catechol (o-quinone)-containing proteins coordinated to particular transition-metal ions by electrochemistry as a model for the marine adhesive interface. Moreover, identification of the possible formation of iron complexes was accomplished by UV-vis absorption spectroscopy. As shown in Fig. S3, a new shoulder peak at 320 nm and absorbance increase at 280 nm was found in water solution containing L-dopa and $\text{Fe}(\text{III})$ as compared to L-dopa alone.

3.3. Detection of peptide oxidative damage induced by Fenton reaction

Based on the above results of tyrosine and L-dopa in solution, a fabricated peptide biosensor was used to detect Fenton reaction. Poly(glu, tyr) (4:1) peptide containing many tyrosine residues was chosen as a model peptide and immobilized on ITO electrode surface via layer-by-layer technique, then the peptide-coated ITO was incubated with the Fenton reagents for required time. The oxidative current of peptide was detected in 3 μM $\text{Os}(\text{bpy})_3^{2+}$. The current response of peptide after incubation with FeSO_4 (1 mM)/ H_2O_2 (4 mM) decreased to 30% of its original signal. This result is in agreement with the results of tyrosine in solution. As shown in Fig. 2, H_2O_2 alone has no effect on the current response of peptide. However, Fe^{2+} alone could cause current response to decrease to certain degree. This may be due to the commonly recognized mechanism (Kehrer, 2000), as shown below:

Fig. 2. Cyclic voltammograms of poly(glu, tyr) (4:1) coated ITO electrodes in 3 μM Os(bpy)₃²⁺ after incubation with (a) H₂O, (b) H₂O₂ (4 mM), (c) FeSO₄ (1 mM), (d) FeSO₄ (1 mM)/H₂O₂ (4 mM), and (e) 3 μM Os(bpy)₃²⁺.

In the above processes, the generation of hydroxyl radicals is less than that with Fenton reaction, therefore resulting in the slight current decrease. Another possible reason might be that the sensitivity on electrode surface is higher than that in solution. These results demonstrated that the peptide on electrode surface could be damaged by hydroxyl radicals. It is suggested that the fabricated biosensor can be used to detect the peptide damage induced by Fenton reaction. Fig. 3 shows that the current response changed as a function of reaction time, and the current response dropped to the lowest in 3 min after incubation with FeSO₄/H₂O₂, much faster than incubation with Fe²⁺ alone. There is little influence on the current response for H₂O₂ alone on the peptide with time, consistent with the above results. Fe²⁺ alone had appreciable effect on the current signal of peptide with time, which dropped to 60% of its original signal after 30 min and the rate of change was much slower than incubation with FeSO₄/H₂O₂. The results for peptide suggest that the current response of peptide on ITO electrode decreased due to tyrosine residue oxidation. Although the oxidized product of peptide with the Fenton reagents could not be characterized, it was most probably that the product was L-dopa. As a result, the catechol group of peptide-bound L-dopa may bind to Fe³⁺ to form chelating

Fig. 3. Plot for the dependence of oxidation current responses of poly(glu, tyr) (4:1) on ITO surface on the incubation time in 3 μM Os(bpy)₃²⁺ after incubation with (a) H₂O, (b) H₂O₂ (4 mM), (c) FeSO₄ (1 mM), (d) H₂O₂ (4 mM)/FeSO₄ (1 mM), and (e) 3 μM Os(bpy)₃²⁺.

Fig. 4. Plot for the dependence of oxidation current responses of poly(glu, tyr) (4:1) coated ITO surface on the concentrations of DFO in 3 μM Os(bpy)₃²⁺ after incubation with (a) H₂O/DFO, (b) H₂O₂/DFO, (c) FeSO₄/DFO, and (d) Fe²⁺/H₂O₂.

complex, which inactivated the electro-activity of catechol group in peptide.

The stability of the biosensor depends on the peptide immobilized on ITO electrodes. Generally speaking, peptides can be kept stable for two weeks, and even longer under anaerobic condition. The reproducibility of the biosensor is shown in Fig. 3 by the error bar of each data point, most of which is in the range of 1–15% for three replicates.

We also investigated the relationship between the electrochemical signal and the concentration of H₂O₂ at a fixed concentration of Fe²⁺ (500 μM). Fig. S4 shows that the current decreased progressively as H₂O₂ concentration was increased, and dropped to the lowest value when the concentration 500 μM. Meanwhile the current response of peptide as a function of concentration of Fe²⁺ was also measured at a fixed concentration of H₂O₂ (500 μM). Similar to the result with H₂O₂, the current response also dropped to the lowest value at 500 μM Fe²⁺ (shown in Fig. S5). From the two figures, it is evident that the peptide was damaged to the greatest extent when the ratio between Fe²⁺ and H₂O₂ is 1:1. In addition, the lowest concentration at which the peptide damage was detected is 10 μM Fe²⁺ or H₂O₂. This concentration is close to the Fe²⁺ and H₂O₂ levels found in vivo.

3.4. Evidence that Fe(III)–L-dopa is present on biosensor

In order to further investigate whether the current signal decrease of peptide is due to the formation of chelating complex between the tyrosine oxidation product and Fe(III), DFO, which is a linear trihydroxamic acid siderophore and can form stable complex with ferric iron (Halliwell, 1989), was incubated with the electrode after reaction with the Fenton reagents in this study. As shown in Fig. 4, the current response of peptide after reaction with the Fenton reagents increased with the increasing concentration of DFO, the degree of increase for peptide reacting with Fenton reagents is higher than that with Fe²⁺ only. Meanwhile, the current response of peptide after incubation with water and H₂O₂ was not affected by DFO significantly. These results indicate that DFO can competitively bind to Fe³⁺ coordinated to the peptide on ITO surface and make the phenol group free to the solution, which results in the increase of current signal again. Therefore, the results provide the strong evidence that current decrease of peptide was due to the formation of Fe(III)–L-dopa complexes on ITO surface.

Fig. 5. XPS N 1s spectral regions of the poly(glu, tyr) (4:1) coated ITO surface after incubation with (a) water and (b) FeSO_4 (1 mM)/ H_2O_2 (4 mM).

3.5. X-ray photoelectron spectral characterization of nitrogen element

In order to exclude the possibility that decrease of current response is not due to the peptide backbone cleavage by hydroxyl radicals, X-ray photoelectron spectroscopy was employed to quantitatively determine the atomic percentage of nitrogen elemental percentage on peptide-coated ITO surface under incubation with water and Fenton reagents, respectively. As shown in Fig. 5, The N 1s peak at 400.2 eV is assigned to nitrogen atoms, present in the amide bonds in the polypeptide chain and amino acid side chains. The N 1s peak at 403.2 eV is assigned to nitrogen atoms, present in the amine group of PDDA layer. There is no difference for the intensity of the two peaks between incubation with the Fenton reagents and with pure water. The atomic percentage of N 1s from PDDA layer after incubation with water and Fenton reagents is $9.4 \pm 0.2\%$ and $8.6 \pm 1.9\%$, respectively, which is $5.3 \pm 0.6\%$ and $4.3 \pm 0.9\%$ for peptide layer. The results show that the atomic percentage of N element on electrode surface in the presence of Fenton reagents has a little change compared to that without Fenton reagents. These results demonstrated that the peptide backbone was not cleaved by the hydroxyl radicals and the peptide damage was induced by tyrosine oxidation. The results further confirm the feasibility of using the electrochemical biosensor to detect protein damage by Fenton reaction.

4. Conclusion

To our best knowledge, it is the first time that an electrochemical biosensor was employed to detect tyrosine oxidation induced by Fenton reaction. Compared to the traditional methods, the method

is sensitive, quick and cheap. As shown from the results, the peptide damage proceed in two steps: oxidation of tyrosine to L-dopa and subsequent formation of Fe(III) -L-dopa complex, which suppressed the electro-activity of L-dopa and resulted in decrease of electrochemical signal. The simple biosensor can detect concentrations of Fenton reagents similar to the level in vivo. We suggest that the biosensor can be used to detect the protein oxidation induced by Fenton reaction.

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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.2010.09.054.

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