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ORIGINAL PAPER

Amperometric biosensor based on tyrosinase immobilized in hydrotalcite-like compounds film for the determination of polyphenols

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Abstract A tyrosinase (Tyr) biosensor has been constructed by immobilizing tyrosinase on the surface of Mg–Al–CO_3 hydrotalcite-like compound film (HTLc) modified glassy carbon electrode (GCE) for the determination of polyphenols. The negatively charged tyrosinase was adsorbed firmly on the surface of a positively charged HTLc/GCE by electrostatic interactions and retained its activity to a great degree. The modified electrode was characterized by cyclic voltammetry and AC impedance spectra. Polyphenols were determined by a direct reduction of biocatalytically generated quinone species. The different parameters, including pH, temperature, and enzyme loading were investigated and optimized. Under the optimum conditions, Tyr/HTLc electrode gave a linear response range of 3–300, 0.888–444, and 0.066–396 μM with a detection limit (S/N=3) of 0.1, 0.05, and 0.003 μM for catechol, caffeic acid, and quercetin, respectively. In addition, the repeatability and stability of the enzyme electrode were estimated. Total polyphenol contents of real samples were also determined to study the potential applicability of the Tyr/HTLc/GCE biosensor.

Keywords Tyrosinase · Mg–Al–CO_3 hydrotalcite-like compound · Biosensor · Polyphenols

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Introduction

The polyphenols, known as vegetable tannins, are plant secondary metabolites generated from plant metabolism, widely existing in the plant bark, roots, leaves, and fruits [1–3]. Polyphenols have unique physical and chemical properties, such as combining with proteins, alkaloids, polysaccharides, and metal ion [4–7]. Moreover, they can be regarded as antioxidant, anti-inflammatory, antiestrogen, antivirutics, antimutagen, anticancerogenics, and play an important role in many fields [8–13]. In view of their wide applications, it is desirable to develop an easy and sensitive analytical method for the low-level polyphenols determination.

Analytical methods such as chromatography [14–17], chemiluminescence [18], capillary zone electrophoresis [19], and spectrophotometric methods [20, 21] are currently employed to determine polyphenols. But these methods require time-consuming, pre-treatment steps, need skilled operators, and are hardly for on-site measurement. Therefore, there is a demand for new analytical technique to determine low concentration of ploypheno1s. Due to the good reproducibility, selectivity, high sensitivity, and easy to miniaturization, electrochemical techniques, especially amperometric biosensors, have been regarded as the best candidates for the detection of polyphenols [22–24]. In this kind of biosensors, the measurement of polyphenols is based on the signal produced by the direct electrochemical reduction of quinones, the product of the enzymatic reaction [25], in the presence of tyrosinase.

The effective immobilization of tyrosinase on the electrode surface is an important step in the development of tyrosinase biosensors. In order to settle this problem, a variety of functional materials have been employed, such as Fe_3O_4 nanoparticles, chitosan, silica sol–gel, polypyrrole,

ionic liquid [22, 26–29], and so on. Finding or synthesizing new materials, which could not only immobilize tyrosinase firmly on the electrode surface, but also retain enzyme's bioactivity, will attract people's attention in quite a long time.

Because of high-potential applications in various fields ranging from ion exchangers, anion adsorbents, drug carriers, catalysts, and membranes [30–34], currently, hydrotalcite-like compounds (HTLcs), or so-called layered double hydroxides, in particular, have become a class of layered materials of strong interest. HTLcs own similar structures with hydrotalcite and can be synthesized by coprecipitation from aqueous solutions of corresponding metal salts, hydrothermal methods, and so on [35, 36]. Due to their positively charged edge-sharing octahedra forming brucite-like host layers, HTLcs not only have large surface areas but also own the ability to immobilize biomolecules depending on their isoelectric point [37, 38]. All the above advantages make them potentially attractive materials as electrode surface modifications and have been applied effectively in building novel biosensors. Mousty et al. [39] reported an amperometric biosensor based on the co-immobilization of laccase from *Trametes versicolor* and a redox active layered double hydroxide to detect the anionic toxic substances and got very low detection limits. Ai et al. [37] reported a glucose biosensor modified with the Ni/Al layered double hydroxide nanoflakes and chitosan for glucose sensing.

Tea polyphenols are the major polyphenolic compounds of tea, which have important health properties and antioxidant activity [40, 41]. In recent days, the studies of tea have attracted great attention because of its health benefits. The content of tea polyphenols detection is one important aspect among these studies. Enzyme-based biosensors have made valuable contributions to the determination of tea polyphenols. According to the literatures, we can find that tyrosinase, laccase, peroxidases, and superoxide dismutase biosensors have been used for the tea polyphenols detection effectively and gained satisfactory results [42–47].

In this work, we constructed a novel electrochemical tyrosinase biosensor for the determination of polyphenols based on Mg–Al–CO_3 HTLc and tyrosinase-modified glassy carbon electrode. By the positively charged HTLc, tyrosinase was adsorbed firmly on the surface of the modified electrode, which not only made the electrode fabrication procedure easy and fast, but also retained the activity of enzyme and enhanced the electrochemical response of polyphenols. The performances of this type of biosensor in determining polyphenols and real samples were investigated. Meanwhile, the parameters such as pH, temperature, and enzyme loading were discussed and optimized in this paper.

Experiments

Reagents

Tyrosinase (EC 1.14.18.1, 5,370 U/mg from mushroom) was purchased from Sigma (USA) and used as received. Catechol was purchased from Shanghai Zhanyun Chemical Co., Ltd. (China). Caffeic acid and quercetin were from Aladdin Reagent Co., Ltd. Phosphate-buffered saline (PBS) was prepared by mixing the stock solutions of 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4 and adjusting the pH with 0.1 M H_3PO_4 or 0.1 M NaOH. Three kinds of Chinese green tea, Longjing tea, Laoshan tea, and Rizhao tea obtained from the local supermarkets, were used as samples. All other chemicals were of analytical grade and used as received without further purification. All the solutions were prepared with redistilled deionized water.

Apparatus

Cyclic voltammetry and differential pulse stripping voltammetry (DPSV) were performed on a BAS Epsilon Electrochemical Analyzer (Bioanalytical Systems, Inc. USA). Electrochemical impedance spectroscopy (EIS) was obtained with a CHI 660C Electrochemical Analyzer (Shanghai Chenhua Co., China). The working electrode was bare glassy carbon electrode (CHI104, d=3 mm) or modified glassy carbon electrode. A Pt wire and an Ag/AgCl electrode were used as the counter and the reference electrode, respectively. A PHS-3C Exact Digital pH meter (Shanghai Kangyi Co. Ltd., China) was used for preparing the buffer solution.

Preparation of Mg–Al–CO_3 hydrotalcite-like compound

The Mg–Al–CO_3 hydrotalcite-like compound was prepared as the method described and characterized by our laboratory [38]. In brief, a 25-mL solution containing 0.06 M Mg$(NO_3)_2$ and 0.02 M $Al(NO_3)_3$ was titrated with 25 mL of the mixture solution of 0.2 M NaOH and 0.02 M Na_2CO_3 under vigorous stirring. The titration rate was 2 mL/min. During the synthesis, the temperature was maintained at 25 °C. The resulting suspension was then aged at 65 °C in a thermostatic bath for 1 h with magnetic stirring. The resulting product was filtered, washed thoroughly with redistilled deionized water, subsequently dried at 90 °C for 24 h in air. The obtained Mg–Al–CO_3 hydrotalcite-like compound was noted as HTLc.

Preparation of Tyr/HTLc modified glassy carbon electrode

Before the surface modification, glassy carbon electrode (GCE) was successively polished with a 0.3 and 0.05 μm

alumina slurry and then rinsed with water, sonicated in ethanol, and redistilled deionized water, respectively. Finally, it was dried under the stream of high-purity nitrogen for further use.

For preparation of enzyme electrodes, a tyrosinase solution was first obtained by dissolving 1.0 mg of tyrosinase in 1.0 mL 0.1 M pH 7.4 PBS, and a HTLc solution was obtained by dissolving 2 mg HTLc in 1 mL redistilled deionized water, then sonicated for 3 h. With a microinjector, 10 μL 2 mg mL^{-1} HTLc solution was deposited on the freshly prepared GCE surface. After the solvent was evaporated, 5 μL of the tyrosinase solution was dropped onto the surface of HTLc/GCE. After drying under ambient condition for 2 h, the tyrosinase-modified electrode was thoroughly rinsed with redistilled deionized water to remove the excess unbound enzyme. When not in use, the thus-prepared enzyme electrode was stored at 4 °C.

Pre-treatment of samples

A 1.0-g green tea of three different kinds obtained from local supermarkets, still sealed in their filter bags, was placed in 6.0 mL of boiling distilled water; after an infusion time of 10 min, 0.5 mL was then taken, allowed to cool at room temperature, and analyzed.

Results and discussion

Characterization of electrochemical behavior of electrodes

Electrochemical behavior of the modified electrodes was first investigated using $K_3Fe(CN)_6$ as a redox probe by cyclic voltammetry. Figure 1 shows the cyclic voltammograms of the (a) bare GCE, (b) HTLc/GCE, (c) Tyr/GCE, and (d) Tyr/HTLc/GCE obtained in 2 mM $K_3Fe(CN)_6$ solution containing 0.1 M KCl. The bare GCE showed a couple of well-defined redox peak with peak-to-peak separation (ΔE_p) of 72 mV (vs. Ag/AgCl). When the electrode was coated with HTLc, an increase in ΔE_p and a decrease in peak currents (I_p) were observed. It indicated that the immobilization of HTLc could decrease the electron transfer rate between electrode surface and Fe $(CN)_6^{3-/4-}$. After a Tyr/HTLc film or Tyr film was modified on the bare GCE, an obvious increase in ΔE_p and decrease in I_p were observed. This phenomenon illustrated that as a biomacromolecular, tyrosinase made the electrode surface insulative and hindered the electron transfer between electrode and electrolyte.

EIS was used to further characterize the modified electrodes. The value of electron transfer resistance (R_{et}) could be obtained from the semicircle diameter which equaled to the R_{et} and varied with different substances

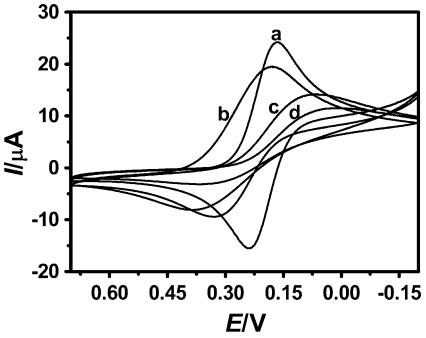

Fig. 1 Cyclic voltammograms of bare GCE (*a*), HTLc/GCE (*b*), Tyr/GCE (*c*), and Tyr/HTLc/GCE (*d*) in 2 mM $K_3Fe(CN)_6$ solution containing 0.1 mM KCl. Scan rate, 50 mV s^{-1}

presented on the electrode surface. The typical morphologies of bare GCE (a), HTLc/GCE (b), Tyr/GCE (c), and Tyr/HTLc/GCE (d) were shown in Fig. 2. A bigger R_{et} was taken on the surface of HTLc/GCE than that at the bare GCE, indicating that the presence of nonconductive HTLc on the surface of bare GCE increased the resistance and obstructed the electron transfer of the electrochemical probe. When the bare GCE was coated with tyrosinase, a bigger R_{et} was obtained which was caused by the hindrance of the macromolecular structure of tyrosinase to the electron transfer. An obvious increase in the interfacial resistance was observed when tyrosinase was on the surface of HTLc/GCE. The increase of R_{et} could be attributed to the synergism of tyrosinase and HTLc. All these results indicated that Tyr/HTLc was successfully immobilized on the surface of the electrode.

Cyclic voltammetric behaviors of catechol

Figure 3 shows the cyclic voltammograms of the bare GCE (a), HTLc/GCE (b), Tyr/GCE (c), and Tyr/HTLc/GCE (d and e) in the presence (a–d) and absence (e) of 0.1 mM catechol at the scan rate of 50 mV s^{-1}. No redox peak was observed at Tyr/HTLc/GCE in the absence of catechol,

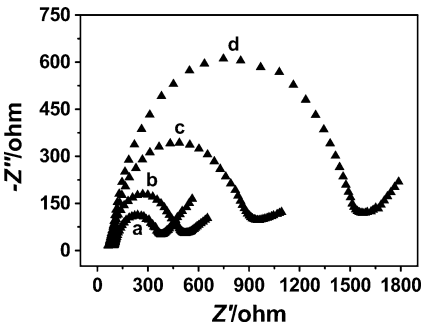

Fig. 2 Electrochemical impedance spectroscopy of bare GCE (*a*), HTLc/GCE (*b*), Tyr/GCE (*c*), and Tyr/HTLc/GCE (*d*) in 5.0 mM Fe $(CN)_6^{3-/4-}$ (1:1) containing 0.1 M KCl with the frequencies swept from 10^5 to 10^{-1} Hz

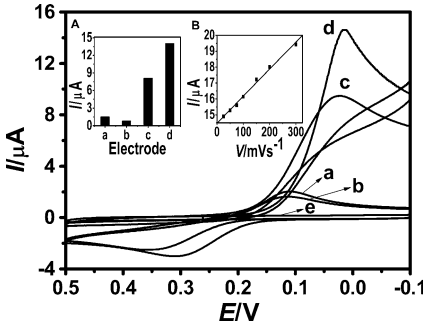

Fig. 3 Cyclic voltammograms of bare GCE (*a*), HTLc/GCE (*b*), Tyr/GCE (*c*), and Tyr/HTLc/GCE (*d*, *e*) in the presence(*a*–*d*) and absence (*e*) of 0.1 mM catechol in 0.1 M PBS (pH 6.6). Scan rate, 50 mV s^{-1}. *Insert*: reduction peak current of different electrodes (*A*), plots for the dependence of reduction peak current on the scan rate of Tyr/HTLc/GCE (*B*)

indicating that the modifier was non-electroactive in the selected potential range. After catechol was added, a decrease in reduction current and an increase in reduction potential were obtained at HTLc/GCE (line b) comparing with the bare GCE (line a), which could be ascribed to the immobilization of nonconductive HTLc on the surface of GCE. The reduction current increased to a large degree when the bare GCE was modified with tyrosinase (line c). After immobilizing tyrosinase on HTLc/GCE (line d), the reduction peak current was 9.2, 17.2, and 1.7 times as much as that obtained at bare GCE, HTLc/GCE, and Tyr/GCE, respectively. Obviously, the observed reduction peak was attributed to the direct reduction of quinone liberated from the enzyme-catalyzed reaction on the electrode surface. The immobilization of HTLc enlarged the loading amount of tyrosinase and increased the current response. The steps of the enzymatic reactions were shown as follows [26]:

$$\text{catechol} + \text{tyrosinase}\,(O_2) \rightarrow \text{o} - \text{quinone} + H_2O \qquad (1)$$

$$\text{o} - \text{quinone} + 2H^+ + 2e^- \rightarrow \text{catechol}\,(\text{atelectrode}) \qquad (2)$$

Inset B in Fig. 3 shows the dependence of reduction peak current on the scan rate. With the increase of scan rate, the reduction peak currents also increased gradually. A linear regression equation for I_p=0.017 v+14.47 (R=0.9968, I_p in μA, v in mV s^{-1}) was obtained, which indicated that the electrode reaction was a typical absorption-controlled process.

With the increase of scan rate, E_p shifted negatively, and a linear equation could be expressed as E_p=0.063–0.048 log (v) (R=−0.952, E_p in V, v in mV s^{-1}). According to the following equation [48]:

$$E_p = K - 2.3030\,(\mathrm{RT}/\alpha nF)\log(v)$$

where E_p (V) is the reduction peak potential of substrate, K is a constant, α is the charge transfer coefficient, n is the electron transfer number, F (C mol^{-1}) is the Faraday's constant, and v is the scan rate. So, the value of RT/αnF can be easily calculated from the slope of E_p–log v. In this work, the slope was −0.048, which equaled to the value of 2.3030 (RT/αnF). Therefore, the values of n could be calculated to be 2.

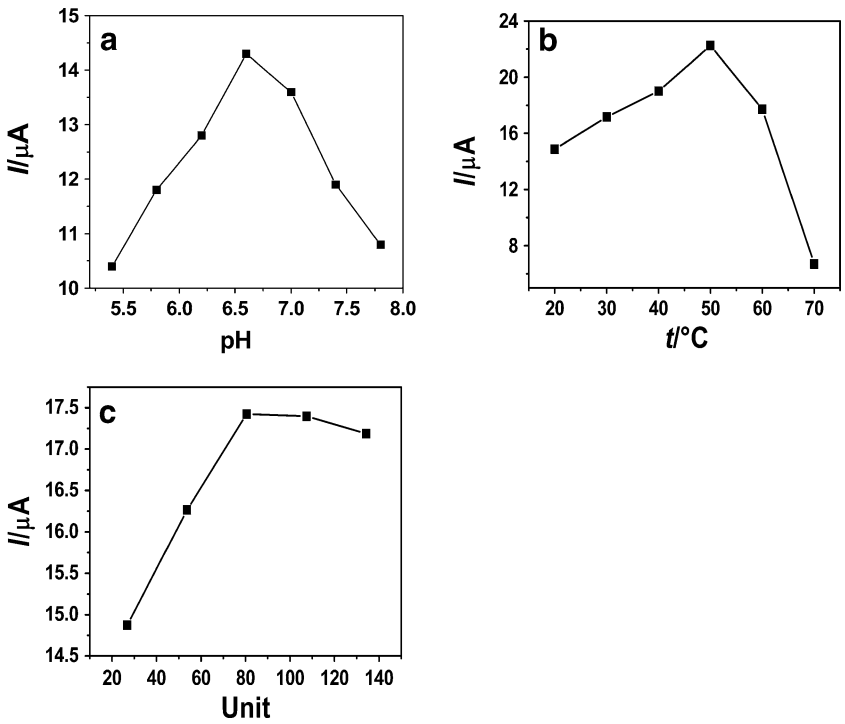

Fig. 4 Effect of pH (**a**), temperature (**b**), and enzyme loading (**c**) on the amperometric response of 0.1 mM catechol in 0.1 M PBS

Optimization of experimental parameters

Effect of pH

The pH dependence of the enzyme electrode was studied between 5.4 and 7.8 in 0.1 M PBS in the presence of 0.1 mM catechol and shown in Fig. 4a. It could be seen that reduction peak current gradually rose with an increasing pH from 5.4 to 6.6. However, when the pH further increased to 7.8, the reduction peak current decreased obviously. This phenomenon should be attributed to the fact that at low pH range, the activity of tyrosinase was improved with an increase in pH, which enlarged the current response, but an excessive high pH reduced the activity of tyrosinase contrarily, causing a decrease in the current response. The optimum biosensor response was achieved at pH 6.6, which was consistent with the optimum pH range of 5–8 reported for free tyrosinase [49]. This indicated that the immobilization procedure did not alter the activity of tyrosinase. In order to obtain maximum sensitivity, pH 6.6 was chosen in subsequent experiments.

Effect of temperature

The temperature dependence of the enzyme electrode was also studied in the range of 20–70 °C in 0.1 M PBS in the presence of 0.1 mM catechol and shown in Fig. 4b. As can be seen, the reduction peak current increased gradually with the changing of the temperature from 20 to 50 °C and reached a maximum at 50 °C, then decreased sharply when the temperature increased further. The above results indicated that at high temperature, the enzyme could be easily denatured. Although the highest response of the biosensor was obtained at 50 °C, in consideration of practical reasons that required the biosensor with high sensitivity and long lifetime, room temperature was selected throughout the experiments.

Effect of enzyme loading

The effect of the tyrosinase loading on the biosensor from 26.85 to 134.25 units was investigated. As shown in Fig. 4c, the response current increased with the amount of the tyrosinase immobilized on the electrode and reached the maximum at 80.55 units and then decreased slightly as the loading of tyrosinase increased more. Therefore, 80.55 units of tyrosinase immobilized on the electrode was chosen for all subsequent biosensors.

Quantitative detection of polyphenols

Quantitative detection of polyphenols at Tyr/HTLc/GCE was investigated. Figure 5 shows the reduction peak current

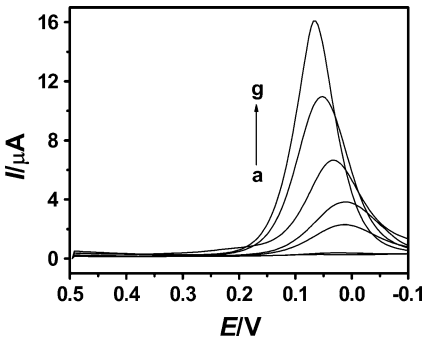

Fig. 5 DPSV obtained at Tyr/HTLc/GCE for solutions of increasing catechol concentration under optimum conditions: *a* 3, *b* 5, *c* 30, *d* 50, *e* 100, *f* 200, and *g* 300 μM

of catechol (vs. Ag/AgCl), which kept on rising with the increasing concentration of catechol in PBS (pH 6.6) by DPSV. However, a negative shift in the reduction peak potential with increasing concentration of catechol was also obtained. This phenomenon can be attributed to the fact that the enzymatic reactions of this experiment were not standard reversible reactions and that the irreversibility of the enzymatic reactions increased with the increased in the concentration of catechol. Caffeic acid and quercetin were also tested to further evaluate the analytical characteristics of the biosensor. Figure 6 displays the calibration curves of Tyr/HTLc/GCE for catechol (a), caffeic acid (b), and quercetin (c) under the optimal conditions. The linear range spanned the concentration of catechol from 3.0 to 300 μM, caffeic acid from 0.888 to 444 μM, and quercetin from 0.066 to 396 μM, with detection limits of 0.1, 0.05, and 0.003 μM and the correlation coefficients 0.999, 0.999, and 0.999, respectively.

Repeatability and stability of the enzyme electrode

The catechol of 5.0 μM was detected to investigate the repeatability of the same Tyr/HTLc/GCE biosensor. A relative standard deviation (RSD) of 6.5% was obtained

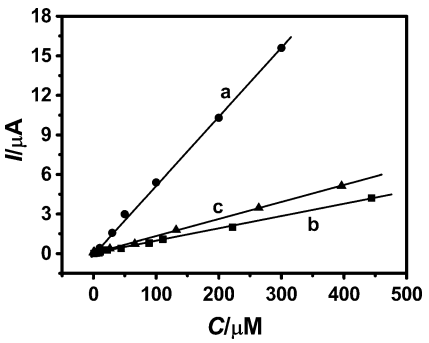

Fig. 6 Calibration curves of Tyr/HTLc/GCE for catechol (*a*), caffeic acid (*b*), and quercetin (*c*) under optimum conditions

Table 1 Estimation of total polyphenol contents in green tea samples by Tyr/HTLc/GCE biosensor and standard method

Samples	Total polyphenol contents (mM; n=8) by sensor-based system	Total polyphenol contents (mM; n=8) by standard method
Rizhao tea	0.032±0.005	0.176±0.010
Longjing tea	0.071±0.009	0.251±0.018
Laoshan tea	0.046±0.006	0.198±0.013

for six successive determinations, which indicated a good repeatability of the measurements with the above-described enzyme electrode. The fabrication reproducibility of four electrodes, constructed independently by the same procedure was also studied. The RSD of 1.07% for 0.1 mM catechol demonstrated the reliability of the fabrication procedure.

The long-term stability of the biosensor was evaluated by measuring the electrode response to 0.1 mM catechol once a week. When not in use, the electrode was stored at 4 °C in a refrigerator. It was found that the biosensor response decreased to 90% after a week, and then decayed to about 82% in 2 weeks. The enzyme electrode retained 72% of its initial value after 1 month indicating the good stability of the biosensor, which can be attributed to the immobilization of HTLc, retaining the enzyme activity.

Application to real samples

For studying the potential applicability of the Tyr/HTLc/GCE biosensor, the total polyphenol contents of three different kinds of green tea obtained from local supermarkets were estimated. The green tea samples were determined after a simple pre-treatment process and analyzed using catechol as standard. The results obtained from the present system and the 4-aminoantipyrine (4-AAP) standard methods were compared and shown in Table 1. Eight replicates of each sample were analyzed to confirm the results. From the results, it could be found that the total content of polyphenols in Longjing tea was higher than that in Laoshan and Rizhao green tea according to the two polyphenols detection methods. Also, it could be seen that comparing with the 4-AAP method, the enzyme-based sensor system measured lower phenolic compound concentrations. The fact that tyrosinase biosensors are restricted to the monitoring of phenolic compounds with at least one free ortho-position and cannot use some phenolic compounds as substrates probably is the reason [50]. However, it was found that an excellent correlation between the results of both methods was obtained, independently of each single tea sample, which made the Tyr/HTLc/GCE biosensor to be an effective and rapid tool for the real samples determination.

Conclusions

In this paper, we fabricated a novel amperometric biosensor based on immobilizing tyrosinase on the surface of Mg–Al–CO_3 HTLc modified electrode for the detection of polyphenols. Tyrosinase was successfully immobilized on glassy carbon electrode with HTLc by electrostatic interactions. What is more, a large amount of tyrosinase loading on the surface of the modified electrode was achieved by the adsorption of HTLc, which led to a good analytical performance for the polyphenols determination. Also, the fabricated biosensor showed low detection limit, satisfactory linear concentration range, excellent stability, high reproducibility, and effective application to real samples. The developed Tyr/HTLc/GCE biosensor will be a new kind of amperometric biosensor for polyphenols detection.

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