Immobilization of Tyrosinase on (3-Aminopropyl)triethoxysilane-Functionalized Carbon Felt-Based Flow-Through Detectors for Electrochemical Detection of Phenolic Compounds

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Abstract

Tyrosinase (TYR) was covalently immobilized onto amino-functionalized carbon felt (CF) surface *via* glutaraldehyde (GA). Prior to the TYR-immobilization, primary amino group was introduced to the CF surface by treatment with 3-aminopropyltriethoxysilane (APTES). The resulting TYR-immobilized CF was used as a working electrode unit of an electrochemical flow-through detector for mono- and di-phenolic compounds (*i.e.*, catechol, *p*-cresol, phenol and *p*-chlorophenol). Additionally, flow injection peaks based on electroreduction of the enzymatically produced *o*-quinone species were detected at −0.05 V *vs.* Ag/AgCl. The resulting TYR/GA/APTES/CF biosensor responded well to all compounds tested with limits of detection range from 7.5 to 35 $nmol^{-1}$ (based on three times S/N ratio). Moreover, such modified electrode exhibits good stability and reproducibility for catechol. No serious degradation of the peak current was found over 30 consecutive injections.

Keywords

Tyrosinase, carbon felt, (3-aminopropyl)triethoxysilane, flow-through detector

1. Introduction

A group of chemicals known collectively as endocrine disrupting compounds (EDCs) are suspected of interfering with the normal function of the endocrine system causing adverse effects in humans and environment. These include a range of synthetic oestrogens, pesticides, plasticizers, and phenolics.[1] With the increasing concern over health and environmental issues, there is a great necessity to detect the environmental pollution such as phenolic compounds. Phenolic compounds are major pollutants in the wastewaters of industry, medical food, and other environmental produces.[2–5] Many of them are very toxic, showing harmful effects on plants, animals, and human health. The development of bioselective detection units for phenols has increased rapidly in recent years.

Analytical methods such as chromatography,[6–9] chemiluminescence,[10] capillary zone electrophoresis,[11–13] and spectrophotometric methods[14] are currently employed to determine phenols. However, time-consuming and low sensitivities limit their applications *in situ*. Therefore, there is an interest in developing simple, sensitive, and effective analytical techniques for their determination. Among them, tyrosinase (polyphenol oxidase) based electrochemical biosensors have the potential to provide a faster, simple, and sensitive method for phenolic compounds assay.[15–23]

Tyrosinase (TYR; polyphenol oxidase, EC1.14.18.1) is a binuclear copper-containing metalloprotein that possesses two different catalytic activities (*i.e.*, phenolase activity, ortho-hydroxylation of monophenols, and catecholase activity, the oxidation of *o*-diphenols to *o*-quinones).[24–26] Based on these activities, a number of TYR-based electrochemical biosensors have been proposed for the determination of mono- and di-phenolic compounds. In particular, the development of highly sensitive biosensors for chlorophenol compounds is an important topic, because chlorophenol compounds are extremely toxic contaminants in ground and surface water. Various immobilization approaches of enzyme such as physical adsorption, covalent binding, encapsulation, entrapment and cross-linking have been proposed. Among them, covalent binding has the advantage that the enzyme is generally strongly immobilized on the surface and unlikely to detach from the surface during repeated use.

Carbon felt (CF) is a microelectrode ensemble of micro-carbon fibre (*cca.* 7 μm in diameter), and possesses a random three-dimensional structure. The CF has high surface area (≈0.1–10 $m^2 g^{-1}$), and shows high conductivity and excellent electrolytic efficiency. Furthermore, the porous structure of CF causes very low diffusion barrier against the solution flow. The CF is an excellent candidate for the working electrode unit of the electrochemical flow-through detector[27,28] compared to other electroactive porous structures.[29–31] On account of these CF characteristics, the novel chemical immobilization strategy of TYR onto the CF surface has been established.

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In this study, the primary amino group ($-NH_2$) was induced onto the CF surface by using APTES. The GA was then covalently immobilized onto APTES-modified CF surface *via* $-NH_2$ of APTES. After that, the TYR was also covalently bonded to CF surface through another aldehyde group of GA. The resulting TYR/GA/APTES/CF biosensor was used as a working electrode unit of biocatalytic enzymatic flow-through detector. The characteristics of the modified surfaces were investigated using field emission scanning electron microscopy (FESEM). The activities of the immobilized TYR toward phenolic compounds were evaluated. Meanwhile, parameters such as GA concentration, applied potential, enzyme immobilization time, and electrolyte pH were discussed and optimized. Operational stability and storage stability were also investigated.

2. Experimental

2.1 Reagents

Tyrosinase (TYR, polyphenol oxidase, EC 1.14.18.1, ≥1000 unit/mg from mushroom) was purchased from Sigma-Aldrich Co., and used as received. (3-Aminopropyl)triethoxysilane (APTES) was obtained from Aladdin Industrial Corporation. Catechol, 4-chlorophenol (4-CP), *p*-cresol, phenol, glutaraldehyde, and toluene were obtained from Sinopharm Chemical Reagent Co., Ltd. A 0.1 M phosphate buffer (prepared using K_2HPO_4 and KH_2PO_4) was used to prepare electrolyte. All reagents were used without further purification. Doubly distilled water was used for the preparation of buffer solution, sample standard solution, and enzyme solution.

2.2 Apparatus

The field emission scanning electron microscopy (FESEM) analysis of bare-CF and the TYR/APTES/GA/CF were performed with a ZEISS (ΣIGMA-HD) microscope. To gain information on the interfacial property of the TYR-modified surface, the electrochemical impedance spectra (EIS) of the fabricated TYR-based CF with electrochemical analyser (CHI 750D, ALS Co. Ltd) was measured. The EIS was performed using deoxygenated phosphate buffer (15 ml, 0.1 mol l^{-1}, pH=7.0) containing $[Fe(CN)_6]^{4-}/[Fe(CN)_6]^{3-}$, 1 mmol l^{-1}. The applied potential was set at the formal potential of $[Fe(CN)_6]^{4-}/[Fe(CN)_6]^{3-}$ redox (*i.e.*, 0.23 V *vs.* Ag/AgCl at pH=7.0). The frequency ranged from 0.01 to 10 kHz. All measurements were performed in air at room temperature (≈20 °C).

Flow injection analysis (FIA) system is composed of a double plunger pump (DMX 2000T, SNK) with a six-way injection valve (SVM-6M2, SNK, 200 µl injection loop) and CF-based electrochemical flow-through detection.[16] All FIA experiments were measured at room temperature. Air-saturated phosphate buffer (0.1 mol l^{-1}, pH=7.0) was used as a carrier. Before the measurements, the carrier solution was flowed at flow rate of 3.0 ml min^{-1} for 1000 s under the applied potential of −0.05 V *vs.* Ag/AgCl to remove weakly adsorbed TYR from the CF surface and to reduce the background current. Then, 200 µl of standard solutions of phenolic compounds were injected, and the cathodic peak currents based on the electroreduction of *o*-quinone species produced by TYR reaction.

2.3 Enzyme immobilization procedures

The CF sheet [from Nihon Carbon Co.] was cut into 10 mm × 3 mm × 3 mm in size (weight, *cca.* 12–13 mg), and washed with doubly distilled water under ultrasonication for 10 min, dried in vacuum for 1 h. The fabricated procedure is similar as previously reported by the authors of this work.[15] Briefly speaking, the CF was dipped into a solution of APTES in toluene, 250 g l^{-1}. After 1 h incubation at room temperature, the CF was washed with toluene under ultrasonication for 2 min and dried in vacuum for 1 h. The APTES-modified CF was immersed in different concentrations of aqueous GA solution (2 ml) and incubated at room temperature for 15 min. The activated CF surfaces were then washed thoroughly with pure water and the CF was placed in enzyme aqueous solution. The activated GA/APTES-functionalized CF was immersed in 2 ml of TYR buffer solutions. After the incubation at 4 °C for 1 h, the CFs were washed with phosphate buffer (0.1 mol l^{-1}, pH=7.0) to remove the weakly adsorbed TYR.

3. Results and discussion

3.1 FESEM measurements of the bare and modified CF

Usually, the activity of immobilized enzyme is significantly influenced by the conformation and structure of enzyme on the matrix surface. In order to obtain the interfacial properties of modified CF surfaces, the FESEM measurements were performed to understand the characteristics of the CF surface. Fig. 1 shows the FESEM images of (A) bare CF, and (B) TYR/GA/APTES/CF electrode. Differing from the bare CF (Fig. 1A), a micrometre-sized, island-like structure and/or film-like structure were observed on the TYR/GA/APTES-modified CF surface (Fig. 1B). This is also evidence of the successful modification on the CF surface.

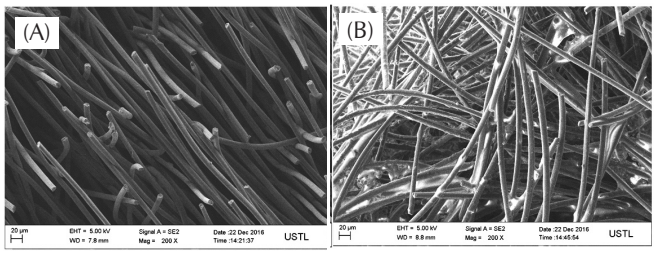

Fig. 1 – Field emission scanning electron microscopic (FESEM) images of (A) bare CF, and (B) TYR/GA/APTES/CF electrode

3.2 Interfacial properties of the bare and modified CF

The electrocatalytic activity and electron transfer properties of the immobilized enzymes on the electrodes are sig-

nificantly affected by the conformation and structure of enzymes on the electrode surfaces. To obtain the interfacial properties of TYR-modified CFs, cyclic voltammogram (CV) was measured by using small redox couples. Fig. 2(A) shows CVs for bare CF (curve a), APTES/CF (curve b), and TYR/GA/APTES/CF (curve c). The APTES/CF showed narrower peak-to-peak separations as compared with TYR/GA/APTES/CF. This is evidence that the structure and morphology of the two CF surfaces are different. APTES/CF showed a much faster electron transfer rate and was in consistency with the EIS data. EIS using small redox couples (*e.g.*, $[Fe(CN)_6]^{4-}$/$[Fe(CN)_6]^{3-}$) is a powerful and common technique for studying the interface properties of surface-modified electrode. Fig. 2(B) shows Nyquist plots of EIS for bare CF (curve a), APTES-modified CF (APTES/CF, curve b), GA/APTES-modified CF (GA/APTES/CF, curve c), and TYR/GA/APTES-modified CF (TYR/GA/APTES/CF, curve d). The electron transfer resistance (R_{CT}) at the electrode surface can be estimated by the Nyquist diameter.[32] The estimated R_{CT} of the four CFs are as follows: bare CF (curve (a) ≈10 Ω), APTES/CF (curve (b) ≈20 Ω), GA/APTES/CF (curve (b) ≈107 Ω), and TYR/GA/APTES/CF (curve (d) ≈255 Ω). The increase in the R_{CT} can be attributed to the different morphologies in the covered area. After tyrosinase was modified on the GA/APTES/CF, the electron transfer resistance evaluated from the diameter of the semi-circle of EIS was 255 Ω. The increase in R_{CT} value indicates the successful modification of tyrosinase. Meanwhile, this result is also in accordance with the FESEM data.

3.3 Optimization of the immobilization parameters using catechol

3.3.1 Effect of GA on the peak current responses of catechol

In this study, catechol was used as a model substrate. TYR catalyses two-electron oxidation of *o*-diphenols to *o*-quinones, which is called catecholase activity in the presence of molecular oxygen.[33] The produced *o*-quinones can be electrochemically reduced to *o*-diphenols at a low overpotential.[34] The effects of GA volume fraction on the peak currents were examined. To understand the importance of covalent bonding by using GA, experiments were carried out by changing the GA volume fraction from 0 to 25 %, as shown in Fig. 3(A). With the increase in GA concentration, the currents also increased to a maximum at 20 % of GA. Higher volume fractions of GA (25 %) resulted in almost the same response. This result indicates that the GA is essential for immobilization of TYR by covalent bonding to detect the catechol. Furthermore, the GA immobilization time upon the peak current of catechol (Fig. 3(A) inset) was investigated. The peak current reached a platform with increasing immobilization time from 15 min to 6 h. Then it decreased while immobilization time increased. It can be concluded that the activity of enzyme was damaged by long reaction time with GA, which is consistent with the result of Fig. 3D.

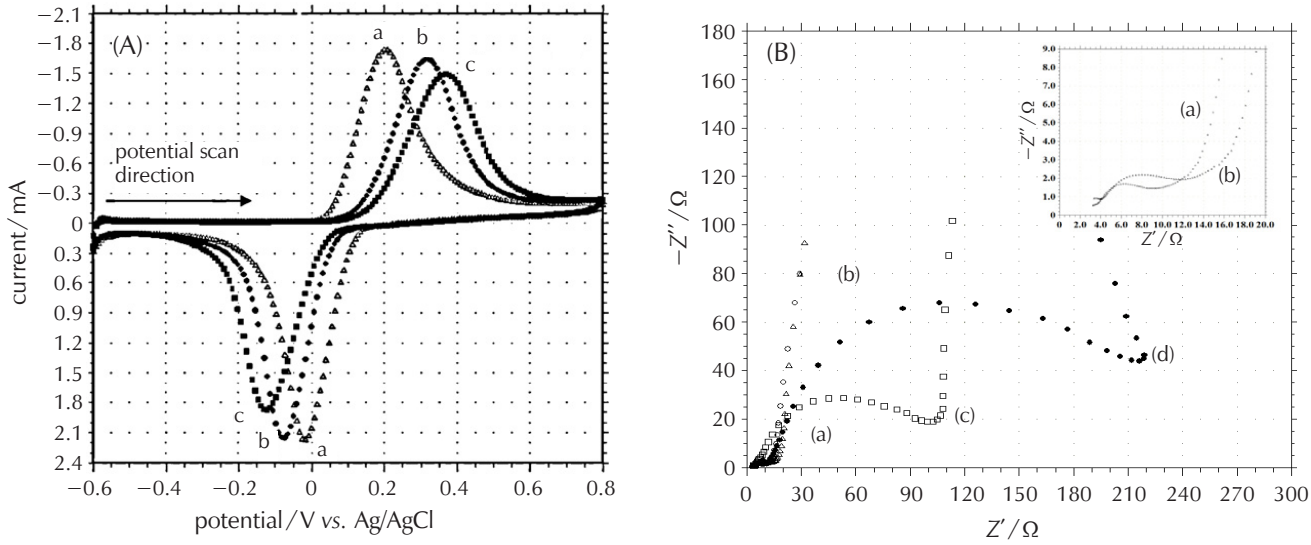

Fig. 2 – (A) CVs of bare CF (a), APTES/CF (b), TYR/GA/APTES/CF (c) in deoxygenized 0.1 mol l^{-1} phosphate buffer (pH=7.0) containing 1 mol l^{-1} $[Fe(CN)_6]^{4-}$/$[Fe(CN)_6]^{3-}$. Scan rate is 50 mV s^{-1}. (B) Electrochemical impedance spectra of bare CF (a), APTES/CF (b), GA/APTES/CF (c), TYR/GA/APTES/CF (d). Electrolyte is the same in panel (A). Applied potential was set 0.23 V *vs.* Ag/AgCl. Amplitude is 0.005 V. The frequency ranges from 0.01 to 10 kHz. Inset shows the enlargement of curves a and b.

3.3.2 Effect of pH in the electrolyte solution on the peak current responses

The influence of the pH of the electrolyte solution on the peak-current response was investigated for a catechol concentration of 10 μmol l^{-1} over the pH range from 5 to 9 using 100 mmol l^{-1} phosphate buffer solution. Fig. 3 (B) shows that the enzymatic activity was dependent on the pH, exhibiting higher activity at pH=6.5. This optimum pH is in good accordance with other biosensors described in the literature.[35] Therefore, pH=6.5 phosphate buffer solution was chosen throughout this study.

3.3.3 Effect of applied potential on the peak current responses

Fig. 3(C) shows the effect of applied potential on the peak current of 10 μmol l^{-1} catechol. Cathodic peak current appeared at +0.15 V and increased with change in the potential from +0.15 to −0.05 V, and the maximum value was observed at −0.05 *vs.* Ag/AgCl. Gradual decrease in peak current in more negative potential region (from −0.1 to −0.2 V) can be attributed to the increased background current, which is probably due to the reduction of dissolved oxygen in carrier. Thus, −0.05 V *vs.* Ag/AgCl was selected as an optimum applied potential of the TYR-CF based flow biosensor.

3.3.4 Effect of TYR immobilization time on the peak current responses

The relationship between the adsorption time of TYR and the peak current responses of catechol (10 μmol l^{-1}) are depicted in Fig. 3(D). The peak current responses were found to be non-dependent on the adsorption time over the range from 30 min to 24 h. The maximum response of 10 μmol l^{-1} of catechol is 1 h. It can be considered that the adsorbed TYR molecule that contributes to the signal generation is adsorbed on the GCE surface during the initial stage of net adsorption processes. This result implies that the adsorption process of the electrochemically active TYR-layer is relatively rapid, and the initial adsorption layer mainly contributes to the generation of the current response. Both short and long immobilization times were not preferable for fabricating the TYR/GA/APTES/CF based biosensor. Therefore, one hour was chosen throughout this study.

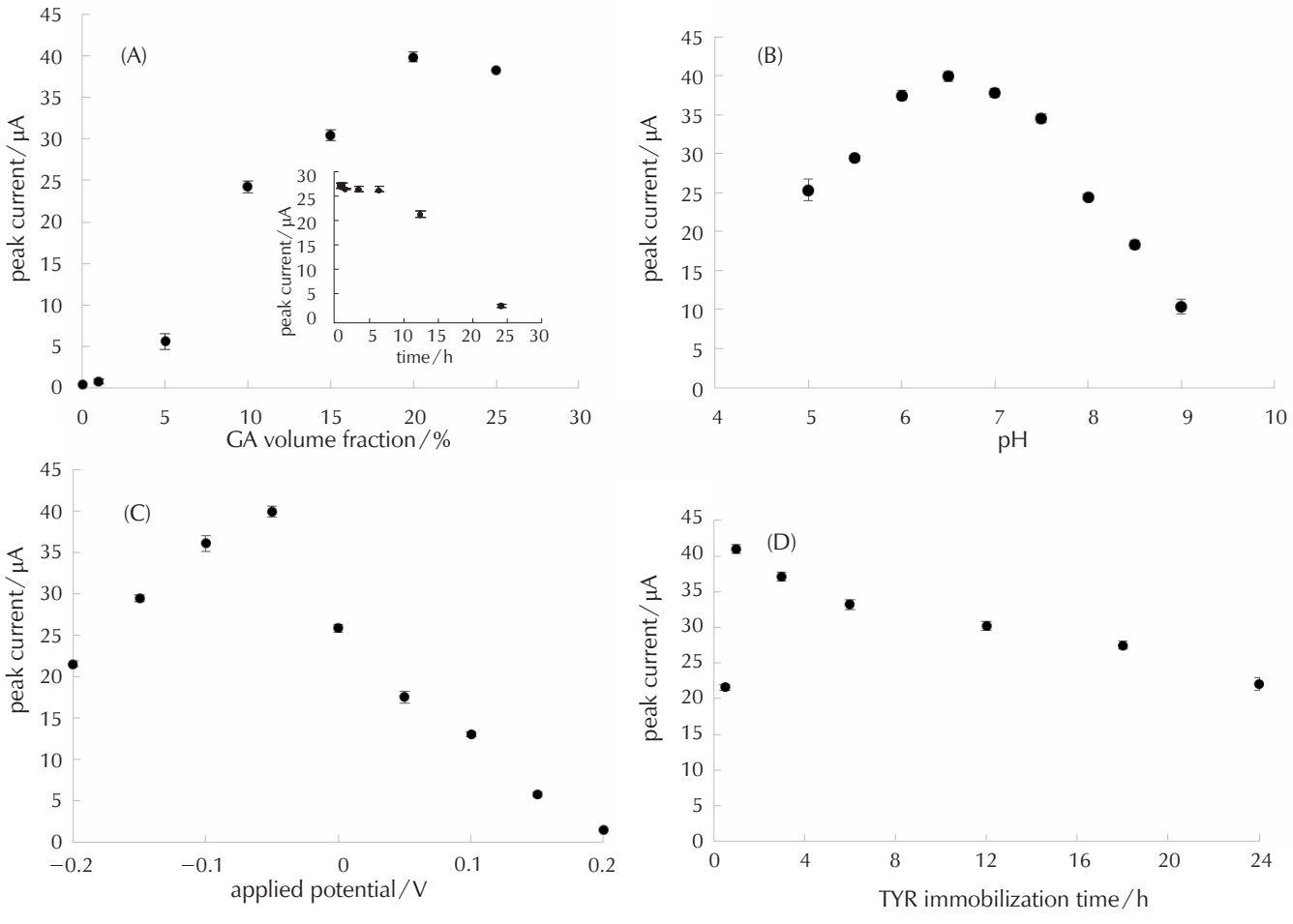

Fig. 3 – Effect of optimized parameters: (A) GA concentration, (B) pH, (C) applied potential, (D) TYR immobilization time on the current response to 10 μmol l^{-1} catechol obtained by TYR/GA/APTES/CF biosensor. Air-saturated 0.1 mol l^{-1} phosphate buffers were used. Applied potential is −0.05V *vs.* Ag/AgCl. Inset in Fig. 3A shows the GA immobilization time dependency.

4. Analytical characteristics of the present biosensor

After optimization of the fabrication parameters above, the analytical properties of TYR/GA/APTES/CF biosensor were subsequently evaluated. Fig. 4 depicts the calibration curves of (a) catechol, (b) *p*-cresol, (c) 4-CP, and (d) phenol obtained by flow injection analysis, which plots the cathodic peak current *vs*. catechol concentration, obtained under certain conditions (applied potential, −0.05 V; carrier flow rate, 3.25 ml min^{-1}; carrier, pH=7.0). The magnitude of the peak-current response by this TYR-based flow-biosensor was linear in the concentration range between 1.0 to 30 μmol l^{-1} with a detection limit of 0.008 μmol l^{-1}, based on the peak current signal-to-noise of 3.

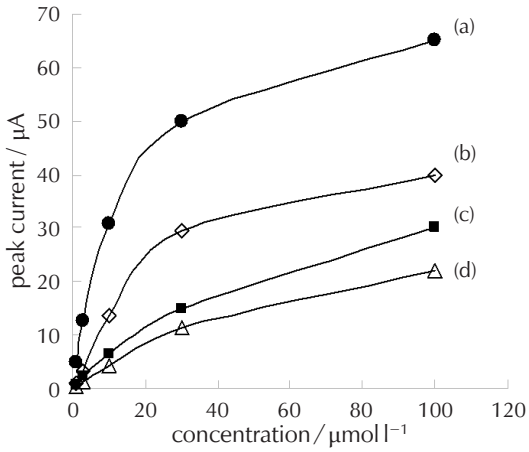

Fig. 4 – Calibration curves of catechol (a), *p*-cresol (b), 4-CP (c), and phenol (d) by the TYR/GA/APTES/CF. Each plot is an average of three measurements.

Table 1 summarizes the performance characteristics of the modified biosensor. The TYR/GA/APTES/CF-based biosensor has the ability to detect low concentration of analytes. Judging from the calibration plots, the fabricated biosensor is useful for detecting not only di-phenolic compounds, but also mono-phenolic compounds, especially 4-CP.

Table 1 – Analytical performances of biosensor

Compound	Sensitivity[a] / μA (mmol l^{-1})$^{-1}$	Detection limit[b] / nmol l^{-1}	Linear range / μmol l^{-1}	Correlation coefficient
phenol	600	35	1.0–30.0	0.9994
catechol	2800	7.5	1.0–30.0	0.9924
p-cresol	1500	14	1.0–10.0	0.997
p-chlorophenol	1000	21	1.0–30.0	0.9909

[a] Slope of the linear portion of calibration curve

[b] Noise level, 7 nA (S/N = 3)

Table 2 summarizes some characteristics of recent covalent bonding-based phenol sensors compared to our sensor. Overall, the device reported here compares favourably with other reported tyrosinase sensors in terms of the parameters outlined. As compared with them, our sensors have the advantages of lower detection limit, higher sensitivity, and good storage stability. It can be concluded that the TYR/GA/APTES/CF-based biosensor has broad specificity toward both mono- and di-phenolic compounds with good characteristics.

5. Operational stability of the TYR/GA/APTES/CF-based flow-through detector

Operational stability is one of the important factors for the practical use of enzyme-based biosensors or as biocatalysts.[36] Fig. 5 displays the typical 30 consecutive flow injection peaks for the sensor with the concentration of 10 μmol l^{-1} catechol. The relative standard deviation (RSD) was 1.85 for 30 successive assays, this is superior to TYR-entrapped carbon paste (RSD = 2.5 %, n = 30).[37]

It can be seen from Fig. 5 that no serious peak degradation was observed over 30 consecutive injections by using catechol as the substrate. It is known that quinone compounds are highly unstable, and easily polymerize and inactivate the TYR.[38] The polymerized product causes the fouling of the TYR-based enzyme electrode surface, lead-

Table 2 – Comparison of the biosensor for the determination of phenolic compounds

Electrode	Analyte	Stability	LOD / μmol l^{-1}	Linear range / mol l^{-1}	Refs.
GCE	catechol	77 % after 7 days	0.021	$5.0 \times 10^{-8} - 1.4 \times 10^{-4}$	41
GCE	phenol	72 % after 30 days	0.2	$4.0 \times 10^{-7} - 1.0 \times 10^{-6}$	42
CPE	phenol	----------	0.1	$2.0 \times 10^{-7} - 15 \times 10^{-6}$	43
GCE	catechol	----------	3.28	$5.0 \times 10^{-6} - 1.2 \times 10^{-4}$	44
CF	catechol	78 % after 25 days	0.008	$1.0 \times 10^{-6} - 3.0 \times 10^{-5}$	this work

GCE – glassy carbon electrode; CPE – carbon paste electrode.

ing to the serious degradation of the response.[39] But in this case, the flow-through system prevents the surface fouling caused by the polymerized products.

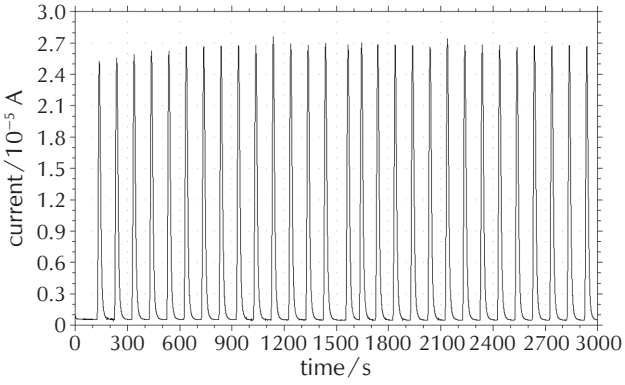

Fig. 5 – Typical peak responses of 30 consecutive injections of 10 μmol l^{-1} catechol obtained with the TYR/GA/APTES/CF biosensor, after 30 days storage in 0.1 mol l^{-1} phosphate buffer (pH=7.0) at 4 °C. The carrier flow rate was 3.0 ml min^{-1}.

6. Storage stability of the TYR/GA/APTES/CF-based flow-through detector

Storage stability is an important factor for the application of immobilized enzymes, because native enzymes usually quickly lose their activity.[40] The relative remaining activity for the determination of catechol over 30 days storage period were checked. The modified electrode maintained 78 % of original activity for catechol after 25 days of storage by checking the activity every 5 days. The results indicate that the TYR/GA/APTES/CF biosensor has good storage characteristics toward the electrochemical detection of catechol.

7. Conclusions

In this study, GA was used to immobilize TYR onto the APTES-modified CF surface. The TYR/GA/APTES/CF biosensor shows excellent results on sensitivity, operational stability, and storage stability for phenolic compounds. The biosensor exhibited excellent operational stability over 30 injections, and maintained 78 % of the original catecholase activity after 25 days of storage. It is also useful for the continuous monitoring of mono-phenolic compounds in our case. Furthermore, this enzyme immobilization strategy would be useful not only for biosensors, but also for biofuel cells.

List of abbreviations and symbols

APTES – (3-aminopropyl)triethoxysilane
CF – carbon felt
CPE – carbon paste electrode
4-CP – 4-chlorophenol
CV – cyclic voltammogram
EDC – endocrine disrupting compound
EIS – electrochemical impedance spectra
FESEM – field emission scanning electron microscopy
FIA – flow injection analysis
GA – glutaraldehyde
GCE – glassy carbon electrode
RSD – relative standard deviation
S/N – signal-to-noise ratio
TYR – tyrosinase
R_{CT} – electron transfer resistance, Ω
Z – electrical impedance, Ω

References
Literatura

1. *T. Corborn, D. Dumanoski, J. P. Myers*, Our Stolen Future, Plume/Penguin Books, New York, 1996, pp. 1–306.
2. *J. Ren, T. F. Kang, R. Xue, C. N. Ge, S. Y. Cheng*, Biosensor based on a glassy carbon electrode modified with tyrosinase immobilized on multiwalled carbon nanotubes, Microchim Acta **174** (3) (2011) 303–309, doi: https://doi.org/10.1007/s00604-011-0616-1.
3. *G. Yong, C. Leone, K. G. Strothkamp*, Agaricus bisporus metapotyrosinase: preparation, characterization, and conversion to mixed-metal derivatives of the binuclear site, Biochemistry **29** (41) (1990) 9684–9690, doi: https://doi.org/10.1021/bi00493a025.
4. *S. Y. Seo, V. K. Sharma, N. Sharma*, Mushroom Tyrosinase: Recent Prospects, J. Agr. Food Chem. **51** (10) (2003) 2837–2853, doi: https://doi.org/10.1021/jf020826f.
5. *J. Švitel, S. Miertuš*, Development of Tyrosinase-Based Biosensor and Its Application for Monitoring of Bioremediation of Phenol and Phenolic Compounds, Environ. Sci. Technol. **32 (6)** (1998) 828–832, doi: https://doi.org/10.1021/es9708934.
6. *D. A. Markham, D. A. McNett, J. H. Birk, G. M. Klecka, M. J. Bartels, C. A. Stapels*, Quantitative Determination of Bisphenol-A in River Water by Cool On-Column Injection-Gas Chromatography-Mass Spectrometry, Int. J. Environ. Anal. Chem. **69** (1) (1998) 83–98, doi: https://doi.org/10.1080/03067319808032576.
7. *A. González-Casado, N. Navas, M. del Olmo, J. L. Vítchez*, Determination of Bisphenol A in Water by Micro Liquid—Liquid Extraction Followed by Silylation and Gas Chromatography – Mass Spectrometry Analysis, J. Chromatogr.

Sci. **36** (11) (1998) 565–570, doi: https://doi.org/10.1093/chromsci/36.11.565.

8. *Q. Liu, W. S. Cai, X. G. Shao*, Determination of seven polyphenols in water by high performance liquid chromatography combined with preconcentration, Talanta **77** (2) (2008) 679–683, doi: https://doi.org/10.1016/j.talanta.2008.07.011.
9. *G. J. Soleas, J. Yan, D. M. Goldberg,* Ultrasensitive assay for three polyphenols (catechin, quercetin and resveratrol) and their conjugates in biological fluids utilizing gas chromatography with mass selective detection, J. Chromatogr. B: Biomed. Sci. Appl. **757** (1) (2001) 161–172, doi: https://doi.org/10.1016/S0378-4347(01)00142-6.
10. *J. X. Du, Y. H. Li, J. R. Lu*, Flow injection chemiluminescence determination of polyhydroxy phenols using luminol–ferricyanide/ferrocyanide system, Talanta **55** (6) (2001) 1055–1058, doi: https://doi.org/10.1016/S0039-9140(01)00452-0.
11. *C. Brage, K. Sjöström,* Separation of phenols and aromatic hydrocarbons from biomass tar using aminopropylsilane normal-phase liquid chromatography, J. Chromatogr. A **538** (2) (1991) 303–310, doi: https://doi.org/10.1016/S0021-9673(01)88851-8.
12. *F. Regan, A. Moran, B. Fogarty, E. Dempsey*, Novel modes of capillary electrophoresis for the determination of endocrine disrupting chemicals, J. Chromatogr. A **1014** (1-2) (2003) 141–152, doi: https://doi.org/10.1016/S0021-9673%2803%2901036-7.
13. *B. Fogarty, F. Regan, E. Dempsey*, Separation of two groups of oestrogen mimicking compounds using micellar electrokinetic chromatography, J. Chromatogr. A **895** (1-2) (2000) 237–246, doi: https://doi.org/10.1016/S0021-9673(00)00716-0.
14. *A. A. García, B. C. Grande, J. S. Gándara*, Development of a rapid method based on solid-phase extraction and liquid chromatography with ultraviolet absorbance detection for the determination of polyphenols in alcohol-free beers, J. Chromatogr. A **1054** (1-2) (2004) 175–180, doi: https://doi.org/10.1016/j.chroma.2004.07.092.
15. *Y. Wang, Y. Hasebe*, Carbon felt-based biocatalytic enzymatic flow-through detectors: Chemical modification of tyrosinase onto amino-functionalized carbon felt using various coupling reagents, Talanta **79** (4) (2009) 1135–1141, doi: https://doi.org/10.1016/j.talanta.2009.02.028.
16. *Y. Wang, Y. Hasebe*, Acridine orange-induced signal enhancement effect of tyrosinase-immobilized carbon-felt-based flow biosensor for highly sensitive detection of monophenolic compounds, Anal. Bioanal. Chem. **399** (3) (2011) 1151–1162, doi: https://doi.org/10.1007/s00216-010-4369-1.
17. *J. Chen, Y. L. Jin*, Sensitive phenol determination based on co-modifying tyrosinase and palygorskite on glassy carbon electrode, Microchim. Acta **169** (3) (2010) 249–254, doi: https://doi.org/10.1007/s00604-010-0320-6.
18. *Y. Xiao, H. X. Ju, H. Y. Chen*, A reagentless hydrogen peroxide sensor based on incorporation of horseradish peroxidase in poly(thionine) film on a monolayer modified electrode, Anal. Chim. Acta **391** (3) (1999) 299–306, doi: https://doi.org/10.1016/S0003-2670(99)00254-8.
19. *E. Dempsey, D. Diamond, A. Collier*, Development of a biosensor for endocrine disrupting compounds based on tyrosinase entrapped within a poly(thionine) film, Biosens. Bioelectron. **20** (2) (2004) 367–377, doi: https://doi.org/10.1016/j.bios.2004.02.007.
20. *L. Tang, G. M. Zeng, J. X. Liu, X. M. Xu, Y. Zhang, G. L. Shen, Y. P. Li, C. Liu*, Catechol determination in compost bioremediation using a laccase sensor and artificial neural networks, Anal. Bioanal. Chem. **391** (2) (2008) 679–685, doi: https://doi.org/10.1007/s00216-008-2049-1.
21. *F. Kheiri, R. E. Sabzi, E. Jannatdoust, H. Sedghi*, Acetone extracted propolis as a novel membrane and its application in phenol biosensors: the case of catechol, J. Solid State Electrochem. **15** (11) (2011) 2593–2599, doi: https://doi.org/10.1007/s10008-010-1250-2.
22. *F. Kaim, A. N. M. Fakhruddin*, Recent advances in the development of biosensor for phenol: a review, Rev. Environ. Sci. Biotechnol. **11** (3) (2012) 261–274, doi: https://doi.org/10.1007/s11157-012-9268-9.
23. *L. M. Kong, S. S. Huang, Z. L. Yue, B. Peng, M. Y. Li, J. Zhang*, Sensitive mediator-free tyrosinase biosensor for the determination of 2,4-dichlorophenol, Microchim. Acta **165** (1) (2009) 203–209, doi: https://doi.org/10.1007/s00604-008-0121-3.
24. *D. E. Wilcox, A. G. Porras, Y. T. Hwang, K. Lerch, M. E. Winkler, E. I. Solomon*, Substrate analog binding to the coupled binuclear copper active site in tyrosinase, J. Am. Chem. Soc. **107** (13) (1985) 4015–4027, doi: https://doi.org/10.1021/ja00299a043.
25. *E. I. Solomon, U. M. Sundaram, T. E. Machonkin*, Multicopper Oxidases and Oxygenases, Chem. Rev. **96** (7) (1996) 2563–2606, doi: https://doi.org/10.1021/cr9500460.
26. *F. García-Molina, J. L. Muñoz, R. Varón, J. N. Rodríguez-López, F. García-Cánovas, J. Tudela*, A Review on Spectrophotometric Methods for Measuring the Monophenolase and Diphenolase Activities of Tyrosinase, J. Agric. Food. Chem. **55** (24) (2007) 9739–9749, doi: https://doi.org/10.1021/jf0712301.
27. *U. Rüdel, O. Geschke, K. Cammann*, Entrapment of enzymes in electropolymers for biosensors and graphite felt based flow-through enzyme reactors, Electroanalysis **8** (12) (1996) 1135–1139, doi: https://doi.org/10.1002/elan.1140081212.
28. *J. González-García, V. Montiel, A. Aldaz, J. A. Conesa, J. R. Pérez, G. Codina*, Hydrodynamic Behavior of a Filter-Press Electrochemical Reactor with Carbon Felt As a Three-Dimensional Electrode, Ind. Eng. Chem. Res. **37** (11) (1998) 4501–4511, doi: https://doi.org/10.1021/ie980144a.
29. *W. J. Blaedel, J. Wang*, Flow electrolysis on a reticulated vitreous carbon electrode, Anal. Chem. **51** (7) (1979) 799–802, doi: https://doi.org/10.1021/ac50043a006.
30. *A. N. Strohl, D. J. Curran*, Flow injection analysis with reticulated vitreous carbon flow-through electrodes, Anal. Chem. **51** (7) (1979) 1045–1049, doi: https://doi.org/10.1021/ac50043a061.
31. *M. Khayyami, G. Johansson, D. Kriz, B. Xie, P. O. Larsson, B. Danielsson*, Flow-injection determination of trace hydrogen peroxide or glucose utilizing an amperometric biosensor based on glucose oxidase bound to a reticulated vitreous carbon electrode, Talanta **43** (6) (1996) 957–962, doi: https://doi.org/10.1016/0039-9140(95)01872-7.
32. *B. Liang, L. Fang, G. Yang, Y. C. Hu, X. S. Guo, X. S. Ye*, Direct electron transfer glucose biosensor based on glucose oxidase self-assembled on electrochemically reduced carboxyl graphene, Biosens. Bioelectron. **43** (2013) 131–136, doi: https://doi.org/10.1016/j.bios.2012.11.040.
33. *J. N. Rodríguez-López, J. Tudela, R. Varón, F. García-Carmona, F. García-Cánovas*, Analysis of a kinetic model for melanin biosynthesis pathway, J. Biol. Chem. **267** (6) (1992) 3801–3810, https://www.ncbi.nlm.nih.gov/pubmed/1740428.
34. *S. Hashemnia, S. Khayatzadeh, M. Hashemnia*, Electrochemical detection of phenolic compounds using composite film of multiwall carbon nanotube/surfactant/tyrosinase on a carbon paste electrode, J. Solid State Electrochem. **16** (2) (2012) 473–479, doi: https://doi.org/10.1007/s10008-011-1355-2.
35. *Y. D. T. Albuquerque, L. F. Ferreira*, Amperometric biosensing of carbamate and organophosphate pesticides utilizing screen-printed tyrosinase-modified electrodes, Anal. Chim.

Acta **596** (2) (2007) 210–221, doi: https://doi.org/10.1016/j.aca.2007.06.013.

36. *J. Tiller, D. Klemm, P. Berlin*, Designed aliphatic aminocellulose derivatives as transparent and functionalized coatings for enzyme immobilization, Des. Monomers Polym. **4** (4) (2001) 315–328, doi: https://doi.org/10.1163/156855501753210808.
37. *J. Wang, F. Lu, S. A. Kane, Y. K. Choi, M. R. Smyth, K. Rogers*, Hydrocarbon pasting liquids for improved tyrosinase-based carbon-paste phenol biosensors, Electroanalysis **9** (14) (1997) 1102–1106, doi: https://doi.org/10.1002/elan.1140091413.
38. *D. A. Robb*, in: R. Lontie (Ed.), Copper proteins and Copper Enzymes, vol. II, CRC Press, Boca Raton, Florida, 1984, pp. 207–240.
39. *F. Ortega, J. L. Cuevas, J. I. Centenera, E. Domínguez*, Liquid chromatographic separation of phenolic drugs using catalytic detection: Comparison of an enzyme reactor and enzyme electrode, J. Pharm. Biomed. Anal. **10** (10-12) (1992) 789–796, doi: https://doi.org/10.1016/0731-7085(91)80082-K.
40. *K. Buchholz, J. Klein*, Characterization of Immobilized Biocatalysts, in: K.V. Mosbach (Ed.), Methods Enzymology, Vol. 135, 1987, Academic Press, London, pp. 3–30.
41. *N. Li, M. H. Xue, H. Yao, J. J. Zhu,* Reagentless biosensor for phenolic compounds based on tyrosinase entrapped within gelatine film, Anal. Bioanal. Chem. **383** (2005) 1127–1132, doi: http://doi.org/10.1007/s00216-005-0115-5.
42. *J. Ren, T. F. Kang, R. Xue, C. N. Ge, S. Y. Cheng*, Biosensor based on a glassy carbon electrode modified with tyrosinase immobilized on multiwalled carbon nanotubes, Microchim. Acta **174** (2011) 303–309, doi: http://doi.org/10.1007/s00604-011-0616-1.
43. *L. W. Wang, Q. Ran, Y, Tian, S. Q. Ye, J. J. Xu, Y. Z. Xian, R. Peng, L. T. Jin*, Covalent grafting tyrosinase and its application in phenolic compounds detection, Microchim. Acta **171** (2010) 217–223, doi: http://doi.org/10.1007/s00604-010-0433-y.
44. *Y. S. Zou, D. Lou, K. Dou, L. L. He, Y. H. Dong, S. L. Wang,* Amperometric tyrosinase biosensor based on boron-doped nanocrystalline diamond film electrode for the detection of phenolic compounds, J. Solid State Electrochem. **20** (2016) 47–54, doi: http://doi.org/10.1007/s10008-015-3003-8.

SAŽETAK

Imobilizacija tirozinaze na pustu od ugljičnih vlakana s (3-aminopropil)trietoksisilanom za protočnu elektrokemijsku detekciju fenolnih spojeva

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Tirozinaza (TYR) je kovalentno vezana na aminiranu površinu pusta izrađenog od ugljičnih vlakana (CF) s pomoću glutaraldehida (GA). Prije imobilizacije tirozinaze primarna amino-skupina uvedena je na ugljična vlakna (3-aminopropil)trietoksisilanom (APTES). CF s imobiliziranom tirozinazom upotrijebljen je kao elektroda u protočnom elektrokemijskom detektoru jednostruko i dvostruko hidroksiliranih fenola (katehol, *p*-krezol, fenol, *p*-klorfenol).

Pri −0,05 V (u odnosu na Ag/AgCl) uočeni su protočni injekcijski signali elektroredukcije o-kinona nastalog enzimskom reakcijom. Biosenzor TYR/GA/APTES/CF dobro se odaziva za sve ispitane spojeve uz detekcijski limit od 7,5 do 35 nmol l^{-1} (tri puta veći signal od šuma). Modificirana elektroda stabilna je i pokazuje dobru reproducibilnost za katehol. Jakost struje signala nije se značajno smanjila ni nakon 30 uzastopnih injektiranja.

Ključne riječi
Tirozinaza, pust od ugljičnih vlakana, (3-aminopropil)trietoksisilan, protočni detektor

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