Effect of Immobilization Methods on Service Life of Tyrosinase Biosensors Developed for Dopamine Monitoring

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Abstract
An effect of immobilization methods on service life of tyrosinase amperometric biosensors was studied where dopamine was selected as a model analyte. Three different immobilization methods were tested such as incorporation of enzyme into the electrode material, immobilization using perfluorosulfonic acid polymer Nafion® and immobilization of enzyme by covalent bonding with the assistance of cross-linking agent glutaraldehyde. All prepared amperometric tyrosinase biosensors were stored dry in refrigerator at 5°C. Finally, the effect of used immobilization was discussed in details.

Key words: Amperometry; Dopamine; Immobilization methods; Storage conditions; Operational stability; Tyrosinase.

Introduction
Amperometric tyrosinase biosensors as bioanalytical devices can find a wide range of applications in clinical analysis, especially in the diagnosis of serious illnesses. The diagnosis is usually based on monitoring of specific biomarkers in body fluids. Among these biomarkers belong organic compounds of low molecular weight (e.g. neurotransmitters), proteins (hormones, enzymes) and even whole cells (microbial pathogens) [1]. The main question remains why tyrosinase biosensors are not used in the clinical practice and still remain an academic subject of interest. An explanation could be found in their short service life which is usually not longer than several weeks [2-8]. It can be considered that the decrease in catalytic activity is probably caused by several factors such as selection of electrochemical transducer (material of working electrode), biorecognition layer (immobilization method used), storing conditions, presence of oxygen etc. An effect of immobilization methods on service life of tyrosinase amperometric biosensors was studied and discussed.

Experimental
Chemicals
Dopamine hydrochloride, 25% glutaraldehyde (GTA), 5% Nafion® in 55% ethanol, lyophilized powder of mushroom (*Agaricus biosporus*) tyrosinase (EC 1.14.18.1), ≥97% and 98% 2-aminoethanethiol hydrochloride (AET) were purchased from Sigma-Aldrich (Prague, Czech Republic). Highly purified water (resistivity >18 MΩ cm) was prepared using purification Milli-Q system from Merck Millipore (Darmstadt, Germany) and phosphate salts needed for prepration of 0.1 mol L^{-1} phosphate buffer solution (PB) were from Lach-Ner s.r.o. (Neratovice, Czech Republic).

Enzyme immobilization
Three different types of immobilization methods were tested. Conventional carbon paste electrode modified with 5% (w/w) tyrosinase enzyme (CPE/Tyr) represents the direct embedding into the electrode material. In this case, 0.4 g graphite powder (particle size >2 μm) from Graphite Týn, spol. s. r. o. (Týn nad Vltavou, Czech Republic), 0.1 g paraffin oil from Merck (Darmstadt, Germany) and 25 mg tyrosinase were homogenized in a ceramic

mortar for 20 min. Resultant paste was pressed into cavity (diameter 3 mm) of Teflon® electrode holder. Electrode surface was renovated by polishing with dry paper after each analysis.

The second strategy for immobilization was the enzyme incorporation into Nafion® membrane (SPCE/Tyr-GTA/Nafion®). A volume of 5 μL of enzyme solution (2.0 mg mL^{-1}) in PB was applied onto surface (diameter 2 mm) screen-printed carbon electrode (SPCE) type C-110 from Metrohm DropSens (Llanera, Spain) and allowed to dry under laboratory conditions. Then, individual enzyme molecules were cross-linked by 10 μL addition of 1% GTA. After 20 min, a volume of 15 μL 1% Nafion® (neutralized by 8 % ammonia solution) was applied directly and allowed to dry under laboratory conditions.

A covalent attachment using cross-linker GTA was used as the last type of immobilization. The gold disk electrode (AuE) of diameter 2 mm was dipped in 10 mM AET solution for 6 hours at 5°C. The modified AuE was washed with deionized water and dried at laboratory conditions. Then, it was immersed into 1% GTA for three hours. Obtained AuE-AET-GTA electrode was transferred into the enzyme solution for one hour (see Fig. 1). Finally, the resulting biosensor (AuE-AET-GTA-Tyr) was washed with redistilled water. Freshly prepared tyrosinase biosensors were stored dry in a refrigerator at temperature 5°C.

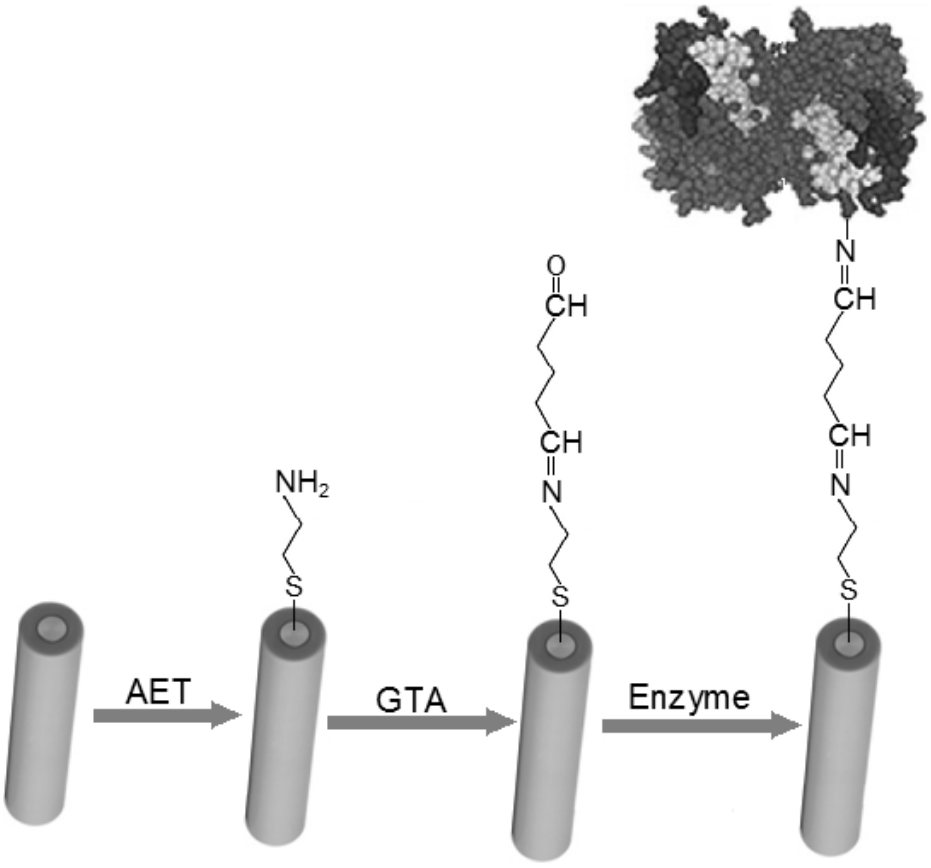

Fig. 1. Individual steps for the covalent cross-linking of tyrosinase on gold disk electrode.

Instrumentation

All amperometric measurements were performed in a typical three electrode configuration consisting one type of tyrosinase biosensors (working) Ag/AgCl/3.0 mol L^{-1} KCl (reference) and Pt wire (auxiliary) electrodes. Resultant setup was always connected to the AUTOLAB PGSTAT101 potentiostat/galvanostat from Metrohm (Prague, Czech Republic), which was operated through Metrohm's NOVA 1.11 software.

Methods

As a supporting electrolyte, non-areated 0.1 mol L^{-1} pH 7.0 PBS was used for each electrochemical measurement due to optimum tyrosinase biocatalytic activity [2]. To characterize the developed biosensor, cyclic voltammetry of 500 µmol L^{-1} dopamine was carried out under following conditions: potential window from -0.4 V to +0.8 V, potential step of 2.5 mV, and scan rate of 10 mV s^{-1}. Amperometric detection (batch configuration) was performed in a conventional glass cell from International Chemistry Co., LTD. (Matsudo-shi, Japan) was usually performed at -0.2 V vs Ag/AgCl/3.0 mol L^{-1} and speed of stirring 400 rpm. Otherwise, any change in the working conditions is described in the legends of the corresponding figures.

Results and discussion

Each of prepared tyrosinase biosensors was characterized using cyclic voltammetry at scan rate 10 mV s^{-1}. Thus, the low rate of scan rate was intentionally set to monitor biocatalysis. For demonstration purposes, Fig. 2A shows typical cyclic voltammograms of 500 µmol L^{-1} dopamine at bare AuE and AuE-AET-GTA-Tyr biosensor respectively. A reduction peak (at +0.1 V) of formed dopamine o-quinone (oxidation product) is more intensive in the presence of enzyme than at bare electrode.

In this work, a working potential of amperometric detection was not optimized. A value of -0.2 V was applied in accordance with the literature [2-4]. Calibration measurements from 10 to 80 µmol L^{-1} dopamine (Fig. 2B) were done at tested biosensors always after three days of storage dry in refrigerator at 5°C to determine the effect of immobilization method used on servise life of tyrosinas biosensor. It was found that biosensors based on covalent bonding had a much shorter lifetime than all others [8]. In contrast to this, an immobilization of tyrosinase enzyme using an incorporation in polymer structure have been able to monitor dopamine concentration for time duration longer than two weeks [4,6,7]. A comparison of electrochemical tyrosinase biosensors developed for dopamine monitoring is shown in Tab. 1.

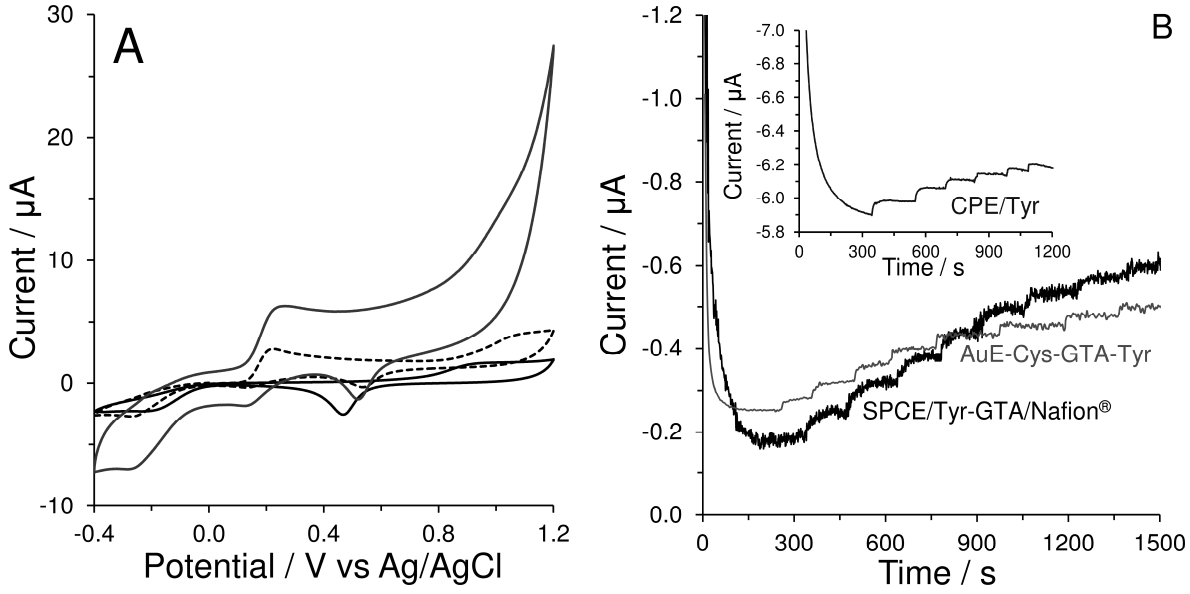

Fig. 2. Cyclic voltammograms of 0.1 mol L^{-1} PB (pH 7.0) (blank; solid black), with 500 µmol L^{-1} dopamine obtained at AuE (dashed black) and at AuE-AET-GTA-Tyr (red) using potential step of 2.5 mV and scan rate of 10 mV s^{-1} (A). Typical amperograms (batch configuration) obtained at fresh CPE/Tyr (blue), SPCE/Tyr-GTA/Nafion® (black) and AuE-AET-GTA-Tyr (red) for several additions of 10 µmol L^{-1} dopamine in 0.1 mol L^{-1} PB (pH 7.0) at working potential -0.2 V and speed of stirring 400 rpm (B).

In 2013, Vicentini et al., comparised the stability of their developed tyrosinase biosensor for monitoring of catechol with the previously reported ones [9]. Surprisingly, they found in literature that an amperometric tyrosinase biosensor utilizing a polyaniline membrane provided stable current response for 120 days [10].

Table I Comparison of electrochemical tyrosinase biosensors for dopamine monitoring.

Biosensors	Storage conditions	Service life	References
Incorporation of tyrosinase into the electrode material (composites)			
CPE/Tyr	dry in refrigerator at 5°C	27.4% after 2 weeks	This work
Immobilization of tyrosinase using polymers			
GCE/Tyr-SWCNTs-Ppy	—	—	[2]
GCE/RGO/β-CD/Tyr/PEI	—	—	[3]
CF-Chit/Tyr/CeO_2/TiO_2	in PB of pH 6.5 at 4°C	75.0% after 2 weeks	[4]
AuE/PEDOT-Tyr	in PB of pH 7.5 at 4°C	35.0% after 5 days	[5]
GCE/Fe_3O_4-Chit-Tyr	dry in refrigerator at 4°C	95.0% after one week	[6]
GCE/AC/Tyr/Nafion®	dry in refrigerator at 4°C	80.9% after 15 days	[7]
SPCE/Tyr-GTA/Nafion®	dry in refrigerator at 5°C	79.5% after 8 days	This work
Immobilization of tyrosinase by covalent bonding			
AuE/CoP-Tyr	PB in refrigerator at 4°C	50.0% after 9 days	[8]
AuE-AET-GTA-Tyr	dry in refrigerator at 5°C	61.3% after one week	This work

Activated carbon (AC); 2-aminoethanethiol (AET); β-cyclodextrin (β–CD); carbonfiber (CF); cobalt (II)-porphyrin film (CoP); glutaraldehyde (GTA); chitosan (Chit) poly(3,4-ethylenedioxythiophene) (PEDOT); polyethylenimine (PEI); polypyrrole (Ppy); reduced graphene oxide (RGO); single-walled carbon nanotubes (SWNTs); tyosinase (Tyr).

Conclusion

Comparison of results obtained with three different amperometric tyrosinase biosensors showed the fact that type of immobilization procedure represents one of decisive factors which determine the service life of tyrosinase biosensors.

Acknowledgments

The support received from the Czech Science Foundation (Project No. 19-03160S) and Faculty of Chemical Technology, University of Pardubice (Project No. SGS-2019-003) are gratefully acknowledged.

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