Flow injection tyrosinase amperometric biosensor with a single-layer graphene modified screen-printed electrode for determination of acetaminophen

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Abstract
The aim of this study was to develop an amperometric biosensor utilizing mushroom (*Agaricus bisporus*) tyrosinase (EC 1.14.18.1) suitable for the selective determination of acetaminophen in human urine. The presented biological device was based on a commercial screen-printed carbon electrode covered with a thin graphene layer (transducer) with an enzyme (bioreceptor) immobilized with glutaraldehyde and Nafion®, and tested on spiked human urine samples.

Key words: Acetaminophen; Amperometry; Graphene; Flow injection analysis; Tyrosinase; Screen-printend electrode; Urine analysis.

Introduction
Acetaminophen, also known as paracetamol, is a common analgesic and antipyretic medication without an anti-inflammatory effect against pain and increased body temperature. As a weak acid, it does not dissociate at most physiological pH values, and therefore penetrates into the tissues only by simple diffusion. Acetaminophen is metabolised in the liver by conjugation to glucuronide or sulphate. These metabolic products are subsequently excreted in the urine, only 2 % in unchanged form. In the overdose, the production of the reactive metabolite of N-acetyl-p-benzoquinone imine (NAPQI) increases. It has been found that at high levels of NAPQI, this metabolite is bound to a -SH group with at least 23 proteins (of which at least 6 are mitochondrial). Histological sections clearly indicate necrotic cell death by centrilobular hepatic necrosis [1]. Thus, it is clear that in clinical analysis, selective determination of acetaminophen in urine is of great importance. Due to the very complex urine matrix, the selectivity of direct voltammetric methods is significantly affected by the presence of interfering substances. The use of sophisticated enzymatic biosensors based on tyrosinase (EC 1.14.18.1) [2], horseradish peroxidase (EC 1.11.1.7) [3] or also aryl-acylamidase (EC 3.5.1.13) [4] can be considered a possible solution. In 2003, a kinetic study of the catalytic oxidation of acetaminophen by the enzyme tyrosinase has been described [5]. In the first step, the acetaminophen is hydroxylated to 4-acetamido-1,2-catechol, which is then oxidized to 4-acetamido-*o*-benzoquinone (4-AOBQ) in the second step. The resulting 4-AOBQ can electrochemically be reduced at a constant voltage, the reaction is accompanied by the exchange of two electrons and two hydrogen ions. In this study, we intend to introduce an amperometric tyrosinase biosensor suitable for flow injection analysis (FIA) using a screen-printed DRP 150 type carbon electrode coated with a thin layer of graphene (DRP-150/Graphene) as a transducer to monitor urinary acetaminophen.

Experimental
Acetaminophen, *N,N*-dimethylformamide (DMF), 25 % glutaraldehyde, 5 % Nafion® in 55 % ethanol, and lyophilized powder of mushroom (*Agaricus biosporus*) tyrosinase (EC 1.14.18.1)

were purchased from Sigma-Aldrich (Prague, Czech Republic). Single-layer of graphene (resistivity ≤0.30 Ω cm; specific surface area 400–1000 $m^2 g^{-1}$) was obtained from ACS Material, LLC (Medford, USA). Highly purified water (resistivity >18 MΩ cm) was prepared using purification system Milli-Q (Millipore) and chemicals needed for prepration of 0.1 mol L^{-1} phosphate buffer solution (PBS) were from Lach-Ner s.r.o. (Neratovice, Czech Republic).

Simple preparation of the DRP-150/graphene amperometric transducer consists in the application of 20 µL dispersion of graphite sheets in DMF (2 mg mL^{-1}) onto a commercial screen-printed carbon electrode (DRP-150 from DropSens, Llanera, Spain) surface under laboratory conditions for 24 hours. The immobilization of tyrosinase on the transducer surface included three separate steps. Firstly, a volume of 5 µL of enzyme solution (2.0 mg mL^{-1}) in PBS was applied and allowed to dry under laboratory conditions for 30 min. This enzyme, which was directly adsorbed onto a thin graphene layer, was then ligated by addition of 1 % glutaraldehyde (3 µL) for 20 min to avoid excessive leaching of the enzyme from the polymer used. Finally, a volume of 10 µL of 1 % Nafion® neutralized by 8 % ammonia solution was applied directly and allowed to dry under the conditions described above for 30 min. If not used, the biosensor was stored in a refrigerator at 5°C.

All electrochemical experiments were performed at tyrosinase biosensor in the wall-jet flow cell (see Fig. 1) obtained from DropSens (Llanera, Spain). The mentioned biosensor was connected to the AUTOLAB PGSTAT101 potentiostat/galvanostat (Metrohm Czech Republic), which was operated through Metrohm's NOVA 1.11 software.

As a supporting electrolyte, non-areated 0.1 mol L^{-1} pH 7.0 PBS was chosen for each electrochemical measurement due to optimum tyrosinase biocatalytic activity [2]. To characterize the developed biosensor, cyclic voltammetry (CV) of 500 µmol L^{-1} acetaminophen was used under following conditions: potential window from -0.2 V to +0.8 V, potential step (E_{step}) of 5mV, and scan rate (ν) of 10 mV s^{-1}. Amperometric detection (AD) in the wall-jet configuration was usually performed at 0 V vs pseudo-reference DRP-150 electrode and flow rate of 0.6 mL min^{-1}. Otherwise, any change in the working conditions is described in the legends of the corresponding figures.

Fig. 1. Methacrylate (transparent) wall-jet flow cell for FIA.

Results and discussion

From electrochemical point of view, the acetaminophen is reversibly anodically oxidized to NAPQI with the participation of 2 e^- and 2 H^+. One oxidation peak at a potential (E_p) +0.375 V and a corresponding reduction peak at +0.318 V was obtained at the transducer in the PBS used (see Fig. 2a). Unlike the tyrosinase biosensor, the oxidation peak at +0.375 V was significantly lower, the reduction peak at +0.318 V completely disappeared, and a new reduction peak at +0.026 V was observed due to the catalytic activity of the enzyme (see Fig. 2b). This new peak corresponds to electrochemical reduction of resulting 4-AOBQ with the participation of 2 e^- and 2 H^+. Evidently, the presence of graphene and tyrosinase allows amperometric scanning at 0 V where no negative interference effects are expected.

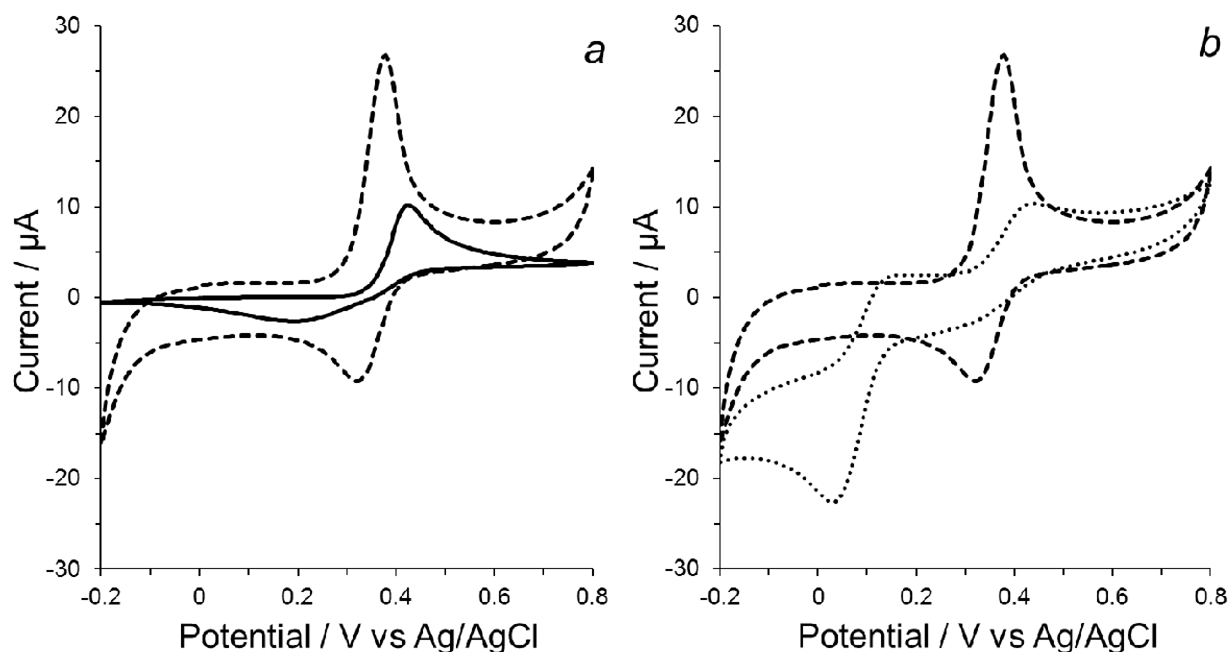

Fig. 2. CV of 500 µmol L^{-1} acetaminophen at bare DRP-150 (solid), DRP-150/Graphene (dashed), and (*b*) DRP-150/Graphene/Tyrosinase-Glutaraldehyde/Nafion® (dotted line) measured in 0.1 mol L^{-1} PBS (pH 7.0) at 10 mV s^{-1}.

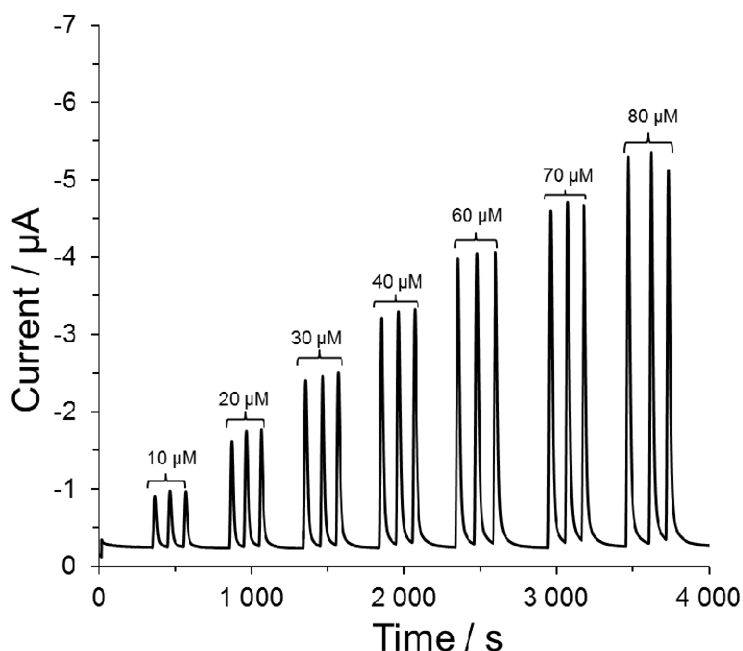

Fig. 3. Typical FIA of acetaminophen at tyrosinase biosensor measured in 0.1 mol L^{-1} PBS (PH 7.0) at working potential -0.1 V and flow rate 0.6 mL s^{-1}.

A freshly prepared tyrosinase biosensor usually provided reproducible results. A typical record obtained during FIA of acetaminophen at -0.1 V is shown in Fig. 3. During the analysis, no enzyme washout was observed. However, it should be noted that the sensitivity greatly decreased with the aging of the sensor. From a practical point of view, there is no problem in using of already prepared transducer and freshly immobilized the tyrosinase enzyme. It is necessary to mention that commercially available DRP-110GPH exists and can be also used as possible transducer.

In our pharmacies, acetaminophen is marketed as Paralen in 500 mg tablets. Assuming that 2 % acetaminophen is excreted unchanged, a limit concentration of ~50 µmol per liter of urine may be considered. Logically, this concentration was used to study the influence of working potential on the selectivity of the developed biological device. A linear range from 5.0×10^{-5} to 1.0×10^{-4} mol L^{-1} (R^2 = 0.9978) with the detection limit (LOD) of 2.34×10^{-6} mol L^{-1} was found for the determination of acetaminophen with amperometric

detection at 0 V. Fig. 4 shows that human urine provides a reduction signal that can distort the final results. Fortunately, negligible influence of the sample matrix was observed at the indicated working potential. Additionally, for repeated injections (N=5) of 50 µmol L^{-1} acetaminophen, values of relative standard deviation (RSD) about 1 % RSD from peak heights obtained for spiked human urine at different working potentials were calculated.

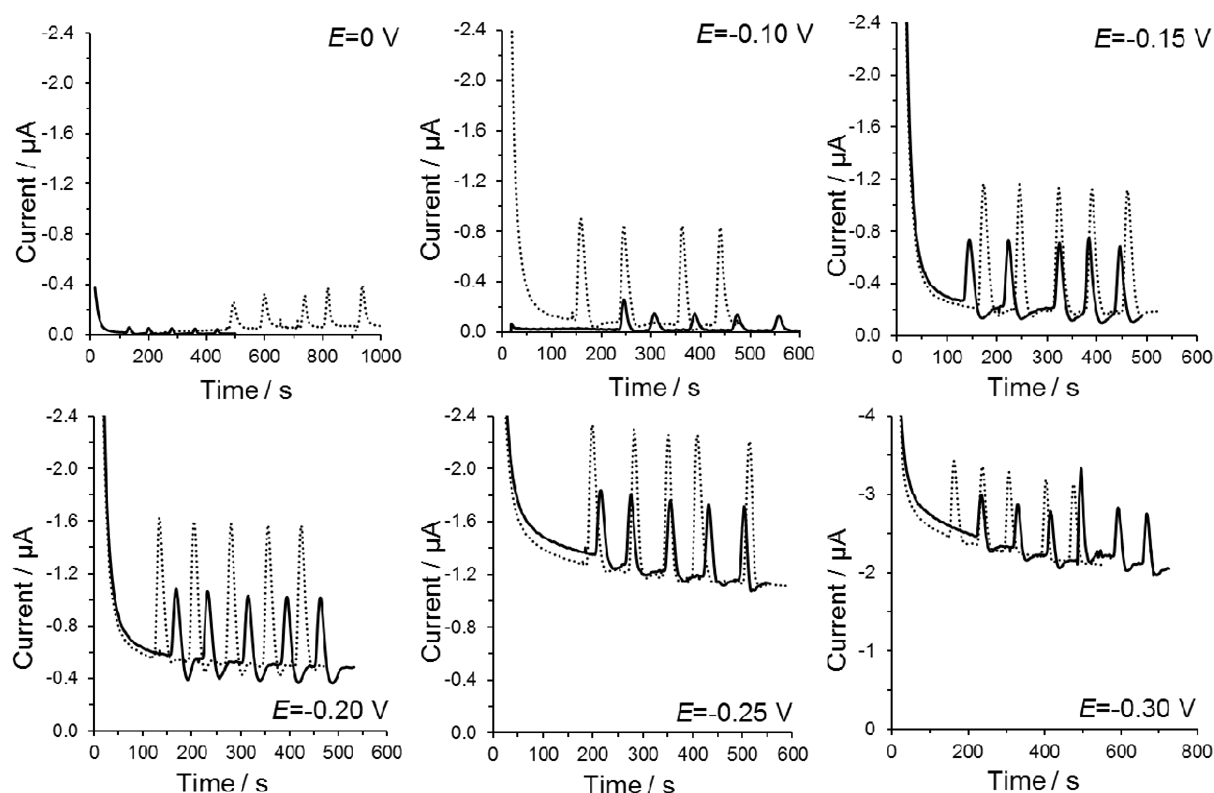

Fig. 4. Effect of working potential on tyrosinase biosensor selectivity. FIA of human urine (solid) and spiked one with 50 µmol L^{-1} acetaminophen (dotted line) at flow rate 0.6 mL s^{-1}.

Conclusion

As shown in this study, the immobilization of tyrosinase onto surface of DRP-150 covered by thin layer of graphene successfully utilized to construct sofisticated biosensor for the selective amperometric determination of acetaminophen in the human urine at working potential 0 V.

Acknowledgments

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