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Electrochemical sensing of trimethylamine based on polypyrrole–flavin-containing monooxygenase (FMO3) and ferrocene as redox probe for evaluation of fish freshness

Sondes Bourigua[a,c], Sarra El Ichi[a,b], Hafsa Korri-Youssoufi[b,*], Abderrazak Maaref[c], Serge Dzyadevych[d], Nicole Jaffrezic Renault[a,**]

[a] Université de Lyon, Laboratoire des Sciences Analytiques, Université Claude Bernard, Lyon 1, 43 Boulevard du 11 Novembre 1918, 69622 Villeurbanne Cedex, France
[b] CNRS UMR-8182, Institut de Chimie Moléculaires et Matériaux d'Orsay, Equipe de Chimie Bioorganique et Bioinorganique, Université Paris-Sud, Bât 420, 91405 Orsay, France
[c] Laboratoire de Physique et Chimie des Interfaces, Faculté des Sciences de Monastir, Avenue de l'Environnement, 5019 Monastir, Tunisia
[d] Laboratory of Biomolecular Electronics, Institute of Molecular Biology and Genetics, National Academy of Sciences of Ukraine,150 Zabolotnogo St., Kiev 03143, Ukraine

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ABSTRACT

Amperometric and impedimetric biosensor for detecting trimethylamine (TMA) which represents good parameters for estimating fish freshness has been developed. The biosensor is based on a conducting polypyrrole substituted with ferrocenyl, where flavin-containing monooxygenase 3 (FMO3) enzyme was immobilised by covalent bonding. FMO3 catalyzes the monooxygenation TMA to trimethylamine N-oxide (TMO). For catalysis FMO require flavin adenine (FAD) as a prosthetic group, NADPH as a cofactor and molecular oxygen as cosubstrate. Ferrocenyl group substituted on the polypyrrole matrix will serve as redox probe for monitoring the response of the biosensor to TMA. The construction of the biosensor was characterized by FT-IR, cyclic voltammetry and impedance measurements. Detection is done through the analysis of the current of oxidation signal of the ferrocenyl groups and compared to the measurement of impedance related to the electrical properties of the layers. Amperometric and impedimetric response were measured as a function of TMA concentration in range of 0.4 $\mu g\,mL^{-1}$–80 $\mu g\,mL^{-1}$ (6.5 $\mu mol\,L^{-1}$–1.5 $mmol\,L^{-1}$). Amperometric measurements show a decrease in current response which is in correlation with the increase of the charge transfer resistance demonstrated by impedance. Calibration curve obtained by impedance spectroscopy shows a high sensitivity with a dynamic range from (0.4 $\mu g\,mL^{-1}$ to 80 $\mu g\,mL^{-1}$). We demonstrated, using ferrocene as redox probe for catalytic reaction of FMO3, that high sensitivity and dynamic range was obtained. The biosensor was stable during 16 days. The biosensor shows high selectivity and its sensitivity to TMA in real samples was evaluated using fish extract after deterioration during storage.

1. Introduction

Fish is one of the most highly perishable food products. During handling and storage, quality deterioration of fresh fish rapidly occurs and limits the shelf life of the product (Sallam, 2007). The quality of fish degrades, due to a complex process in which physical, chemical and microbiological forms of deterioration are implicated. Because of consumer demand for fresh refrigerated foods with extended shelf life, considerable research has been directed toward using various preservation technologies to preserve or prolong the shelf life, while ensuring the safety, of fresh foods, including fishery products (Siripatrawan et al., 2009). So the quality of fish product is influenced by its freshness. Therefore, the rapid and accurate determination of freshness is desired for quality and processing controls (O¨zogul et al., 2005). Many indicators were used for the assessment of fish quality during storage (Gill, 1992). Among all of them, trimethylamine (TMA) is one of the volatile compounds that can be measured in order to estimate fish freshness (Pacquit et al., 2006). It is produced by the decomposition of trimethylamine N-oxide (TMAO) by microorganisms and its concentration rapidly increases in products after the death of microorganisms (Hamada-Sato et al., 2005).

Flavin containing monooxygenase 3 (FMO3) is the enzyme that breakdown trimethylamine (TMA) into trimethylamine oxide (TMAO) through a process called N-oxygenation. The flavin-containing monooxygenase 3 is an NADPH-dependent enzyme that

* Corresponding author. Tel.: +33 1 69154723; fax: +33 1 69157281.
** Corresponding author. Tel.: +33 4 72448306; fax: +33 4 7231206.
E-mail addresses: hafsa.korri-youssoufi@u-psud.fr (H. Korri-Youssoufi), nicole.jaffrezic@univ-lyon1.fr (N. Jaffrezic Renault).

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catalyzes the oxygenation of a wide variety of nucleophilic compounds containing sulfur, nitrogen or phosphorus atoms. Oxidation of TMA is catalyzed by FMO3 enzyme with NADPH as a co-factor following the reaction.

$$TMA+NADPH+H^+ + O_2 \xrightarrow{FMO3} TMAO + H_2O + NADP^+$$

The FMO enzymes metabolize xenobiotic substrates, converting the substrate to a more polar product, aiding in its excretion from the body. They demonstrate broad substrate specificity and have considerable overlap in function with the cytochrome P450 enzymes. The FMO oxygen transfer potential is much lower than that of the cytochrome P450 enzymes, limiting FMO substrates to heteroatom nucleophiles with the oxidative reaction resulting in the formation of N-oxides. The FMO3 mechanism differs from that of cytochrome P450 enzymes by binding and activating molecular oxygen prior to binding of the substrate (Cashman, 2005). NADPH binds to the FMO3 enzyme and reduces the TMA to TMAO.

Various analytical techniques have been employed for the determination of TMA. These techniques include gas chromatography (Mushiroda et al., 1999), Colorimetric assay (Pena-Pereira et al., 2010), flow injection analysis (Dhaouadi et al., 2007; Sadok et al., 1996) or detection using cell (Gamati et al., 1991; Li et al., 1994) or recombinant cell (Suska et al., 2009). Biosensors based in catalytic reaction of FMO3 has also been developed for TMA (Mitsubayashi et al., 2004) and others volatiles compounds (Mitsubayashi and Hashimoto, 2002). Catalytic oxygenation reaction of such compounds by FMO3 leads to better sensitivity than the others kinds of TMA biosensors (Fillit et al., 2008). The problem in enzymatic biosensors is the lost of activity when the enzyme is immobilised on solid state beside their low stability with time (Sheldon, 2007). Conducting polymers (CPs) such as polypyrrole and polyaniline have been demonstrated as a good matrix for the construction of enzymatic biosensor as they preserve the catalytic activity of the enzyme. CPs provides a controlled method of localizing an enzyme in a defined area on the electrodes (Ahuja et al., 2007).

In this work we report the development of an electrochemical biosensor for TMA detection, based on FMO3 enzyme, flavin-containing monooxygenase, immobilised on electropolymerized functionalised polypyrroles with ferrocene. The ferrocene group could play the role of redox probe as it electrochemical response is sensitive to any modification in their environment. Amperometric biosensor from the response of the ferrocene will be measured and compared to the impedance measurement of the polypyrroles layer after such reaction. The performances of the new biosensor were evaluated for the detection of TMA in PBS solution and in real sample. Different parameters have been investigated to determine the optimum working conditions.

2. Experiments

2.1. Reagents

The gold electrodes were fabricated by evaporated gold layer of 300 nm thickness on silicon substrate using titanium as adhesion layer of 30 nm thickness. These gold electrodes were provided by the LAAS, CNRS Toulouse. Flavin-containing monooxygenase 3 was purchased from Sigma and is a human recombinant microsomes (EC 1.14.13.8), 2.5 mg/mL protein with specific activity of 44,000 units/mg protein. It was stored in solution of 100 mM potassium phosphate buffer, pH 7.4. Trimethylamine (25 wt.% in water) was purchased from Sigma–Aldrich.

The monomer 3-N-hydroxyphtalimide-pyrrole (py-NHP) was synthesized from 3 acetic acid methyl ester following the procedure described in a previous work (Godillot et al., 1996). The product was obtained after various synthesis steps. The first step is the acetylation reaction of N-protected pyrrole followed by its conversion to 3 acetic-acid methyl ester using thallium based oxidative transposition. The hydrolysis of 3-acetic acid methyl ester in basic condition gives the 3-acetic acid pyrrole with a 70% yield. Grafting of N-hydroxyphtalamide was realised using N,N′ dicyclohexyl carbodiimide which leads to 3-acetate N-hydroxyphtalimido pyrrole (Py-NHP) with a 60% yield. The purity of product was characterized by NMR and mass spectrometry. 1-Ethyl amino-ferrocene was synthesized by reduction of cyanomethyl ferrocene (purchased from Aldrich) using mixed metal hybride halide $LiAlH_4$–$AlCl_3$ (1:1), following a procedure described in our previous work (Godillot et al., 1996). The product obtained with a 60% yield and was characterized by NMR and mass spectrometry.

The buffer solution used for all experiments was phosphate buffer saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, 0.01 M KH_2PO_4 and 0.01 M K_2HPO_4, pH 7. All solutions were made up in ultrapure water (resistance 18.2 MΩ cm^{-1}) produced by a Millipore Milli-Q system.

2.2. Preparation of a copolymer poly (N-hydroxyphtalimide-pyrrole-co-pyrrole)

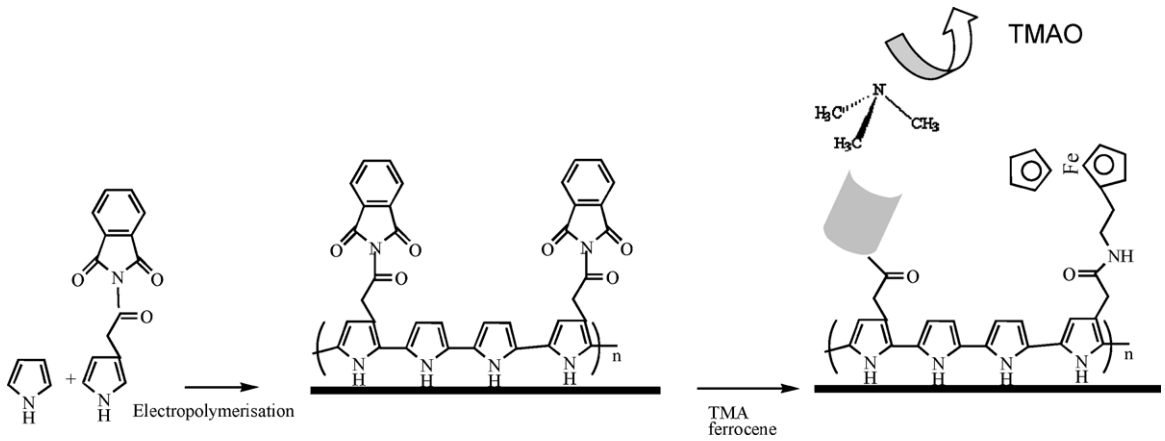
In order to control the thickness of the copolymer film and then the reproducibility of polypyrroles layers chronoamperometry technique was chosen for the film electrodeposition on the gold electrode. Before electropolymerization, the gold electrode was first polished using diamond slurries (6, 3, 1, 0.25 μm, respectively). The electrode was washed in an ultrasonic bath for 10 min in water and ethanol and dried under a dry N_2 flow. After cleaning, the gold electrodes were immediately placed as working electrode in a three-electrode electrochemical cell at room temperature. The copolymer poly(N-hydroxyphtalimide-pyrrole-co-pyrrole) film was electrochemically prepared on gold electrode from a solution containing 8 mM pyrrole, 2 mM N-hydroxyphtalimide-pyrrole and 0.5 M $LiClO_4$ as supporting electrolyte in acetonitrile. The concentration was chosen according to the previous study where we demonstrate that the film obtain with this ratio of concentration allows a less steric hindrance for protein immobilisation (Lê et al., 2010). The solution was degassed with nitrogen for 10 min prior to use. The potential applied for the electropolymerization reaction was 0.9 V/SCE and was stopped when we reach the desired charge of 25 mC cm^2. After electropolymerization, the electrodes were washed with acetonitrile and water.

2.3. Enzyme attachment

The enzyme, FMO3 was attached to the polymer surface by covalent binding of the amine group from lysine to the N-hydroxy phtalamide reactive group by the formation of amide link. The copolymer-modified electrode was immersed after electropolymerization in an enzyme solution of 0.5 mg mL^{-1} FMO3 in a phosphate buffer solution (8 mM, pH 7.0) and was left overnight at +4 °C. The electrode was then washed with a phosphate buffer solution pH 7.0 to remove the unbound enzyme.

2.4. Covalent attachment of ethyl amine ferrocene

After immobilization of the enzyme FMO3 onto the copolymer layer, the electrode was dipped for 1 h at a controlled temperature of 20 °C in a solution of phosphate buffer pH 8.5 containing ethyl amine ferrocene. The pH of 8.5 was used in order to avoid the protonation of the amine on the ferrocenyl group. This solution was realised by dissolving 1 mg of the ethyl amine ferrocene in 10 μL of acetonitrile and diluted in 100 μL of PBS pH 8.5. The electrode was rinsed with a pH 7.0 phosphate buffer solution and stored at 4 °C until use.



Scheme 1. Representation of the chemical process in the formation of the biosensor based on functionalised polypyrrole.

2.5. TMA extraction from fish

200 g of a sample of horse-mackrel, merlin, were kept at 25 °C for 12 h and then brown with moltar before being stirred with water during 1 h. The resulting suspension solution was centrifuged. The obtained supernatant solution of the fish extract was then filtered and kept in the freezer at −20 °C until use. The presence of TMA in sample was confirmed by gas chromatography coupled to the mass spectroscopy using ionisation impact. Concentration of the extract was measured by HPLC. HPLC was realised using reversed phase column of C18 and the mobile phase was the mixture of acetonitrile–water in gradient elution mode, with the flow rate of 1 mL/min. Meanwhile, the acetonitrile concentration in the mobile phase was linearly increased from 0 to 60% at 2.5 min, the mobile phase composition was kept constant until 3.5 min, and then the percentage of acetonitrile was increased to 70% at 10 min and to 100% at 15 min.

2.6. Fourier-transform infrared spectroscopy (FT-IR)

A Brucker IFS 66 spectrometer with MCT detector was used to acquire infrared spectra. The spectra was recorded in reflectance mode with Attenuated Total Reflectance (ATR) technique using a germanium crystal. The film was deposited onto the gold surface and the spectrum was recorded before and after reaction with the protein FMO3.

2.7. Cyclic voltammetry (CV)

Cyclic voltammetry and amperometric measurements were done in a conventional three electrode cell configuration consisting of a working gold electrode (0.11 cm^2), a saturated calomel reference electrode (SCE) and platinum plate (0.5 cm^2) as a counter electrode. All cyclic voltammetry experiments were performed using a Voltalab 80, model PGZ 402, controlled by Voltamaster 4 software. The measurements were performed in a 8 mM solution of PBS buffer at pH 7.

2.8. Electrochemical impedance spectroscopy (EIS)

Three electrode cell configuration was used for EIS measurements. The impedance analysis was performed with a Voltalab model PGZ 402 impedance analyzer in the frequency range between 100 mHz and 100 kHz, and with an amplitude of alternative voltage of 10 mV. Measurements were performed in a PBS buffer solution at pH 7.0. The measured impedance spectra were analysed in terms of electrical equivalent circuits using the analysis program Z view.

3. Results and discussion

3.1. Construction of the biosensor and its characterisation

The electrodeposition of the copolymer poly(N-hydroxyphtalimide-pyrrole-co-pyrrole) onto the surface of the gold electrode was monitored using chronoamperometry technique. The Chronoamperogram shows an initial abrupt increase of the recorded current due to the diffusion controlled monomer oxidation in solution. It is followed by a slight increase in current due to an electrochemical nucleation of oligomers produced in solution and growth of copolymer onto the gold electrode. The schematic steps for the formation of the biolayer and the enzyme recognition are shown in Scheme 1. After the electrodeposition of the copolymer film, the electrode surface became rich with activated ester (py-NHP). This functionalised surface is able to form covalent binding to enzyme and ethyl amine ferrocene by their reaction with terminal amine group (NH_2) of both protein and ferrocenyl group. The modified electrode was analysed by FT-IR to determine the efficiency of their covalent attachment. Spectra were recorded after polymer formation and after protein attachment (figure not shown). Spectra of polypyrrole bearing activated ester shows the band assigned to polypyrrole backbone at 1631, 1564 and 1475 cm^{-1} corresponding to C=C stretching vibrations and broad bands at 1182 and 1134 cm^{-1} assigned for N–C stretching band. Beside well defined pyrrolidinedione stretching vibration of C=O are observed at 1818 and 1784 cm^{-1} which is the signature of the activated ester groups functionalised polypyrrole (Korri-Youssoufi et al., 2001). After the covalent attachment of protein FMO3 and ferrocenyl group new stretching band appear at 1526 cm^{-1} associated with the amide band of the protein and at 1650 cm^{-1} associated with the amide bond of the ferrocenyl group.

Modified electrode was electrochemically characterized in phosphate buffer solution at pH 7.0 by cyclic voltammetry. Fig. 1 shows the gold surface modified by the copolymer layer after covalent attachment of FMO3 and ferrocenyl groups. The covalent attachment of ferrocenyl group lead to the appearance of two peaks, where the anodic is at 0.45 V and the cathodic at 0.25 V attributed to the redox couple of ferrocenyl group. The intensity of redox wave confirms the high electroactivity of ferrocene groups attached to conducting polypyrrole.

Nyquist plot of the gold surface modified with the poly(N-hydroxyphtalimide-pyrrole-co-pyrrole) and after FMO3 and ethyl

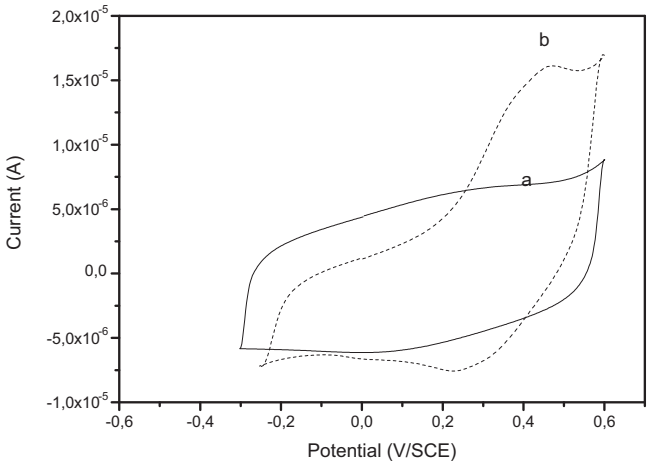


Fig. 1. Cyclic voltammograms corresponding to the various layers grafted onto the gold electrode: (a) poly(py-NHP-co-py), and (b) poly(Py-FMO3- ferrocene-co-py), scan rate of 0.1 V/S.

amine ferrocene attachment are shown in Fig. 2. The Nyquist plot shows that the interfacial impedance increases after enzyme immobilization on polypyrrole layer. Attachments of the ethyl amine ferrocene on the copolymer layer leads to a decrease of interfacial impedance which prove that ethyl amine ferrocene increase the conductivity of the layer. The impedance spectra were fitted with a Randles circuit, shown in Fig. 2B which gives an excellent fit to the experimental data. This equivalent electrical circuit consists of resistive and capacitive elements: Rs is the solution resistance, Rf is the film resistance, Cf is the film capacitance, The constant phase element CPE is then related to the space charge capacitance at the poly(pyrrole-FMO3- ferrocene-co-pyrrole)/electrolyte interface, Rch is related to the charge transfer resistance at the poly(pyrrole-FMO3- ferrocene-co-pyrrole)/electrolyte interface. The constant phase element W is the Warburg impedance due to mass transfer to the electrode surface.

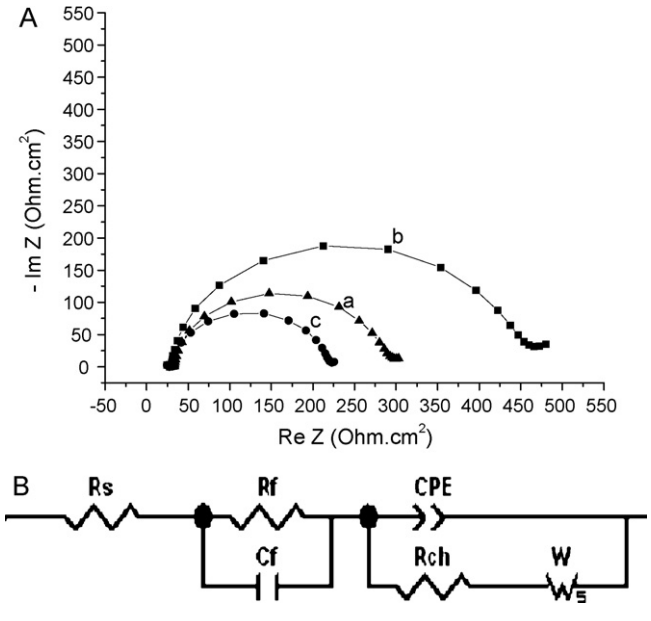


Fig. 2. Nyquist diagram ($-Im(z)$ vs. $Re(z)$) for the non-faradic impedance measurements in 8 mM PBS pH 7.0 solution) corresponding to the various layers grafted onto the gold electrode: (a) poly(py-NHP-co-py), (b) poly(py-FMO3-co-py, and (c) poly(Py-FMO3- ferrocene-co-py). Spectra were obtained between 100 mHz and 100 KHz. Amplitude of Alternative voltage: 10 mV.

3.2. Response of the biosensor to TMA substrate

The detection of TMA is performed by following the signal of the oxidation of the redox ferrocenyl groups. This later reacts as redox probe which is sensitive to the variation of chemical properties of the layer. In fact the enzymatic reaction of FMO3 involves the cofactor NADPH and transform it to $NADP^+$. The $NADP^+$ remains bound to the enzyme FMO3 and molecular oxygen binds to the prosthetic group and forms the peroxyflavin species. Monooxygenation of the substrate and release of a molecule of water leaves the flavin in its oxidized state. The $NADP^+$ molecule dissociates and the enzyme is ready for another catalytic cycle (see Eqs. (1)–(4)) (Phillips and Shephard, 2008).

$$FAD + NADPH + H^+ \xrightarrow{-2e^-} FADH_2 + NADP^+ \quad (1)$$

$$FADH_2 + O_2 \rightarrow FADH\text{–}OOH \quad (2)$$

$$FADH\text{–}OOH + Me_3N \rightarrow FADH\text{–}OH + Me_3NO \quad (3)$$

$$FADH\text{–}OH \rightarrow FAD + H_2O \quad (4)$$

The presence of the $NADP^+$ species on the biolayer modified their electrical properties and then the electrochemical oxidation of ferrocenyl group. Amperometric biosensor response corresponds to the measurement of current oxidation of ferrocenyl group as electrochemical probe. TMA oxidation was monitored by analysis of the oxidation of ferrocenyl group before and during TMA transformation. Cyclic voltammograms of enzymatic electrodes in the absence and presence of trimethylamine were then analysed (Fig. 3A) and showed a decrease in the current value of the oxidation peak of ferrocenyl group after 3 min of catalytic reaction of TMA with the biosensor. The current decrease is depending on the TMA concentration in the sample.

Different amounts of TMA varying between 2.5 and 50 $\mu g\,mL^{-1}$ were tested with the biosensor. Upon addition of the TMA, for all the modified electrodes, the current decreases continuously as a function of TMA concentration (Fig. 3B). The response of enzyme modified electrode formed with poly(pyrrole-FMO3- ferrocene-co-pyrrole) to TMA was found to be linear in the range of 2.5–10 $\mu g\,mL^{-1}$ with regression coefficient of 99%. The sensitivity calculated from the slope of the calibration curve is evaluated to 6 $(\mu A/cm^2)/\mu g\,mL^{-1}$. The detection limit of 0.4 $\mu g\,mL^{-1}$ was calculated taking into account the signal to noise ratio of 3. This value is lower than the detection limit demonstrated in others biosensors of TMA based on FMO3. For example, within the catalytic reaction of FMO3, the TMA was monitored by measuring the oxygen consumed during the reaction by clark oxygen electrode and a detection limit of 1 mM (59 $\mu g\,mL^{-1}$) (Mitsubayashi et al., 2004) was demonstrated.

A highly sensitive amperometric biosensor could be designed thanks, firstly, to the developed approach consisting in immobilizing the FMO3 enzyme on the polypyrrole matrix which preserves its catalytic properties and, secondly, to the use of feroocenyl groups as redox probe.

Trimethylamine–FMO3 catalytic reaction was monitored by impedance spectroscopy as this analytical method could be more sensitive than amperometric measurements (Tlili et al., 2008). Measurements have been achieved with varying frequencies from 100 mHz to 100 kHz in PBS solution at pH 7. Systematically, the modified electrode was equilibrated with a range of concentrations of the TMA from 0.4 to 160 $\mu g\,mL^{-1}$. The impedance spectra (Nyquist plot) obtained after addition of different concentrations of TMA are shown in Fig. 4A where $Re(z)$ is the real part and $Im(z)$ is the imaginary part of the complex impedance Z.

Immediately, at low frequencies, the impedance modulus $|Z(f)|$ increases clearly with the increase of TMA concentration, which indicates that a larger amount of TMA has reacted on the surface.

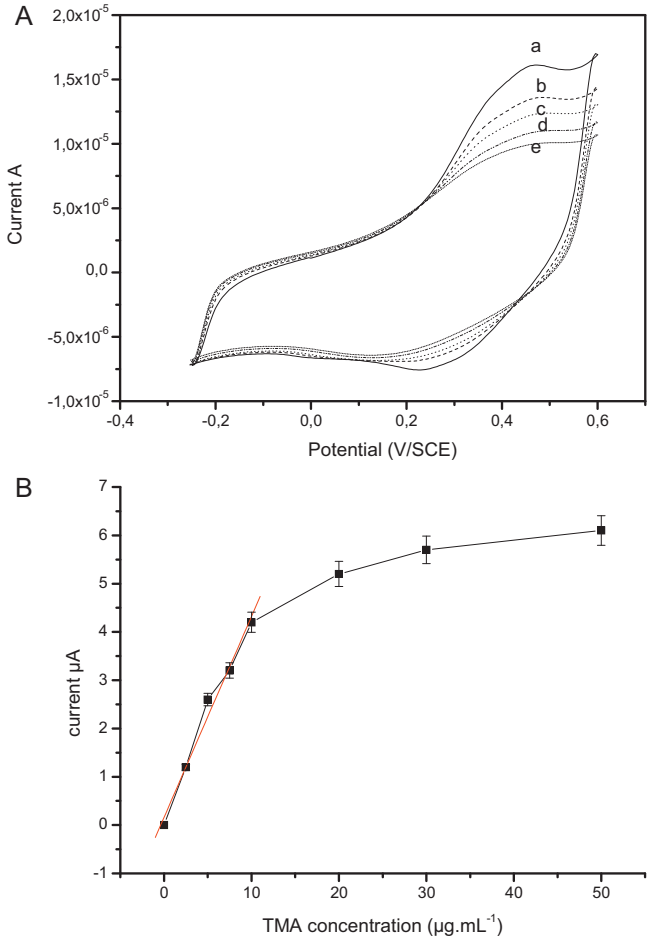


Fig. 3. (A) Cyclic voltammograms of poly(Py-FMO3- ferrocene-co-py) in the absence (a) and (b–e) presence of various concentration TMA (b) 5 µg mL^{-1}, (c) 10 µg mL^{-1}, (d) 20 µg mL^{-1}, (e)50 µg mL^{-1}. The measurement was realised in PBS solution at scan rate of 0.1 V/s (B) The relationship between the measured current and the trimethylamine concentration at fixed potential of 0.45 V/SCE.

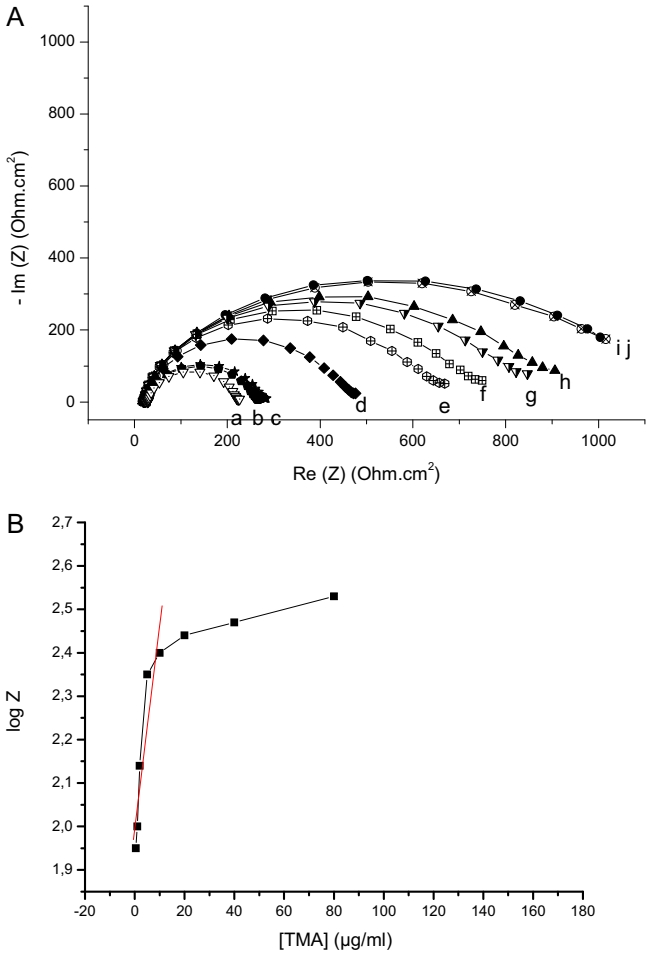


Fig. 4. (A) Nyquist plot of poly(Py-FMO3- ferrocene-co-py) during reaction of various trimethylamine concentration in solution PBS pH 7.0, (a) 0 µg mL^{-1} TMA; (b) 0.4 µg mL^{-1} TMA; (c) 1 µg mL^{-1} TMA; (d) 2 µg mL^{-1} TMA; (e) 5 µg mL^{-1} TMA; (f) 10 µg mL^{-1}; (g) 20 µg mL^{-1} TMA; (h) 40 µg mL^{-1} TMA; (i) 80 µg mL^{-1} TMA; (j) 160 µg mL^{-1} TMA. Spectra were recorded in the frequency range between 100 mHz and 100 KHz, using a modulation voltage of 10 mV. (B) Calibration curves of the TMA biosensor obtained from impedance spectra.

This reaction leads to a decrease in the kinetic of electron transfer of the redox probe ferrocenyl group to the electrode which induces an increase in the charge transfer resistance of the layer which is in concordance with the decrease in amperometric measurement. The calibration curves for TMA detection obtained by measuring the value of modulus $|Z(f)|$ at frequency range of 0.5 Hz where the maximum variation was obtained was shown in Fig. 4B. Linear range for TMA detection was obtained from 0.4 µg mL^{-1} to 10 µg mL^{-1} with coefficient regression of 0.95 which is the same as previous amperometric measurements. However enhanced of dynamic range of TMA detection, corresponding to the response of the sensor before it reach saturation, was obtained by the impedance method which is observed in the range of 0.4–80 µg mL^{-1}. This range is larger than that obtained in the case of amperometric detection and also for conductimetric TMA biosensor developed previously which was from 2 to 40 µg mL^{-1} (Fillit et al., 2008).

3.3. Optimisation of biosensor

In order to optimize the sensitivity of electrochemical measurement for detection of TMA, the effect of various parameters on the amperometric biosensor response was analysed. The effect of the concentration of enzyme solution was investigated by varying the FMO3 enzyme concentrations from 0.5 to 1 mg mL^{-1} during the covalent attachment. Results show that a larger linear range of the trimethyleamine detection is obtained with the concentration of FMO3 of 0.5 mg mL^{-1} during the biosensor set-up. The excess of the immobilised protein may leads to enzyme agglomeration and then to a limited access to its active site.

The effect of the total phosphate concentration in buffer solution on the response of the enzyme electrode was determined by keeping the pH value at 7.0. The concentration effect of the buffer solutions was studied between 4 and 32 mM. The optimum working value of the buffer solution was found as 8 mM.

Enzymes are proteins that become denatured at high temperatures. The effect of the temperature on the activity of the FMO3 enzyme in this biosensor was determined using 8 mM phosphate buffer at pH 7.0. The response of the enzyme electrode was measured at temperatures between 10 and 40 °C. The immobilised FMO3 in polypyrrole layer showed maximum activity at 20 °C. The thermal denaturation of the enzyme caused a decrease in the response of the enzyme electrode for higher temperatures.

3.4. Reproducibility and storage stability of the biosensor

The reproducibility of the biosensor and the response of the enzyme electrode were investigated within 6 modified electrodes and a standard deviation of 11% was obtained. Comparing the reproducibility of our sensor to those described in literature, the same value was obtained with potentiometric biosensor based

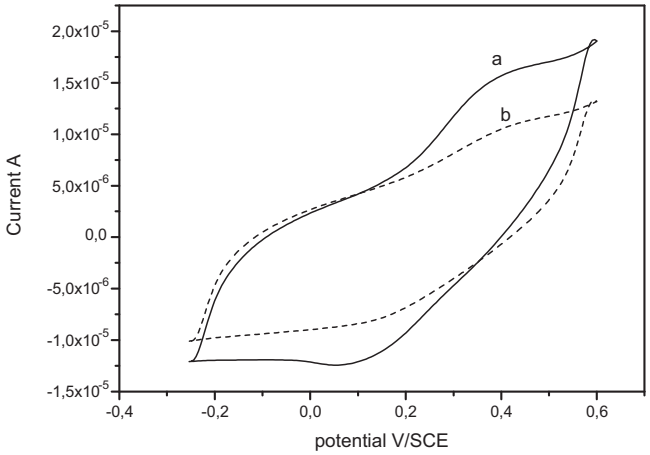


Fig. 5. Cyclic voltammograms of poly(Py-FMO3- ferrocene-co-py) in the absence (a) and (b) with extract of 0.5 mL of TMA diluted in 5 mL of PBS solution.

on microbial sensor where relative standard deviation was determined to be 14% (Gamati et al., 1991). High reproducibility are generally obtained with volatile compounds when derivatization procedure was performed which maintain the amine more stable and less likely to be lost as gases during measurement and storage (DaCosta et al., 1990). These methods of detection are not direct and need the chemical reaction before measurement by coupling volatile amine with other products such as 9-fluorenylmethyl chloroformate or ethyl bromoacetate. High reproducibility was also obtained when flow system was used where the concentration of volatile compound was maintained stable during measurement (Mitsubayashi et al., 2004). The reproducibility obtained within this sensor using direct method without any derivatization procedure and any flow system is acceptable for a volatile compound.

The operational lifetime of the biosensor, defined as the period during which the sensitivity remains constant, was also investigated by checking periodically with the same sensor, its amperometric sensitivity for 10 $\mu g\,mL^{-1}$ of TMA. The biosensor shows a decrease of 10% of it response after 5 days and remains stable during two weeks.

3.5. Evaluation of fish freshness

The evaluation of fish freshness was realised by measuring the extract sample from horse-mackerel fish. The unknown concentration of 500 μL extract solution was diluted in 5 mL PBS solution and measured within the biosensor. Fig. 5 shows the results obtained after 5 min of the reaction of the fish extract sample with the biosensor. Decrease in redox activity of ferrocenyl group was observed as demonstrated previously with TMA sample. The amperometric analysis allows evaluation of the concentration of TMA extract to 2 $\mu g\,mL^{-1}$.

To confirm the presence of TMA in fish extract analysis of fish sample was performed by GC/MS and compared to pure TMA. GC shows a high peak obtained at retention times of 1.4 in both sample pure TMA and fish extract corresponding to retention time of TMA. This peak is the most important observed in the GC curve of fish extract compared to the other obtained peaks. The identification of volatile amine in fish sample was confirmed by the presence of ion fragment at m/z of 58 for TMA in both TMA sample and in fish extract (see figure in supplementary information).

To evaluate the average recovery of such sensor for TMA evaluation in fish sample. The TMA was evaluated by HPLC as another method. The TMA in fish extract was measured with the present sensor and compared with the value obtained with HPLC. For this purpose, a sample of fish extract with unknown TMA concentration was used for the measurement and the obtained value was compared to the calibration curve determined by measuring known concentrations of TMA between 1 and 10 $\mu g\,mL^{-1}$. TMA in fish sample was found to be $1.9 \pm 0.1\ \mu g\,mL^{-1}$. Comparative value of TMA measured with present sensor of 2 $\mu g\,mL^{-1}$ against HPLC as reference method demonstrates average recovery of 105%.

4. Conclusion

In this study, we have described, the development of a new enzyme biosensor for trimethylamine detection based on the covalent attachment of the flavin containing monooxygenase and ferrocenyl groups to an electropolymerized polypyrrole film. We demonstrated that the ferrocenyl groups are able to act as redox probe and monitor the catalytic reaction of FMO3 to TMA. Amperometric biosensor of TMA was obtained leading to a sensitivity of 6 $\mu A\,\mu g\,mL^{-1}\,cm^{-2}$. Monitoring the TMA by electrochemical impedance spectroscopy shows the same sensitivity and improves the dynamic range of TMA detection. In fact, a low detection limit of 0.4 $\mu g\,mL^{-1}$ of TMA and a dynamic range from 0.4 to 80 $\mu g\,mL^{-1}$ were obtained by impedance.

This novel biosensor for TMA analysis with high sensitivity, rapid response and large dynamic range seems to be a very promising analytical tool for the evaluation of fish freshness in real samples.

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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.2011.07.005.

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