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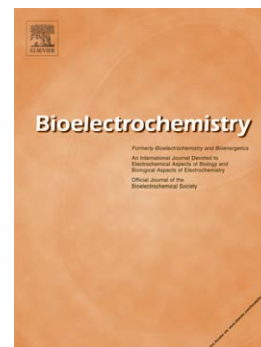
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Maciej Klis, Jerzy Rogalski, Renata Bilewicz

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VOLTAMMETRIC DETERMINATION OF CATALYTIC REACTION PARAMETERS OF LACCASE BASED ON ELECTROOXIDATION OF HYDROQUINONE AND ABTS

Maciej Klis^a, Jerzy Rogalski^b and Renata Bilewicz^{a,}*

^a Faculty of Chemistry, University of Warsaw, Pasteura 1, 02-093 Warsaw, Poland

^b Department of Biochemistry, Maria Curie Skłodowska University, Skłodowskiej Sq 3, Lublin 20-031, Poland

Abstract

A convenient method for the measurement of the catalytic activity of laccase is proposed based on the voltammetric determination of catalytic reaction substrates: 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) diammonium salt (ABTS) and 1,4-benzenediol (hydroquinone). The measurement performed using microelectrodes working under spherical diffusion conditions is both accurate and simple, and allows to monitor parallelly the consumption of substrate and formation of product of the catalytic reaction. The method proposed in this paper was compared with the two generally employed procedures based on oxygen measurement by Clark electrode and on spectrophotometry. The procedure described in the present paper was found to be simpler and more reproducible results were obtained than using Clark electrode. Compared to spectrophotometry a larger range of catalytic reaction substrates can be studied including colourless compounds.

Key words: laccase, microelectrode, Michaelis-Menten model, catalytic activity, enzymatic kinetics, ABTS, hydroquinone

* Renata Bilewicz

Faculty of Chemistry, Warsaw University

Pasteura 1, 02-093 Warsaw, POLAND

e-mail: bilewicz@chem.uw.edu.pl

Tel: +48 22 8220211 ext. 345

Fax: +48 22 8224889

Introduction

Laccases, EC 1.10.3.2, p-diphenol: dioxygen oxidoreductase, belong to the group of multicopper enzymes, including e.g. ascorbic acid oxidase and ceruloplasmin. Laccase was first described by Yoshida [1], and was characterized as a metal containing oxidase by Bertrand [2]. This makes it one of the oldest enzymes ever described [3].

Laccases catalyze the oxidation of a broad range of substrates e.g. polyphenols, substituted phenols, diamines, but also some inorganic compounds [4]. They are widely distributed in nature and can be grouped into two categories, plant and fungal laccases. Laccase or laccase-like activity has been demonstrated in higher plants, in some insects and bacteria. The best-known laccases are of fungal origin, especially those belonging to the class of white-rot fungi.

Laccases of plant origin are reported to play an important role in wound response and lignin synthesis [5], whereas in fungi they are involved in lignin degradation [6] as well as in several other functions, including pigmentation, fruiting body formation, sporulation, and pathogenesis [4]. Degradation of lignin, a major component of wood, is very important for the global carbon cycle. White-rot fungi are believed to be the only microorganisms able to selectively and efficiently degrade native lignin [7].

In addition to the strictly biological functions, laccases are being investigated for a variety of practical applications ranging from use in the pulp and paper industry to their possible use in bioremediation, analytical processes, and organic synthesis. Due to capability to polymerize or depolymerize aromatic substrates, laccase has a number of biotechnological applications that include waste water treatment [8], biopulping and textile biobleaching [9]. Considerable concern recently has been expressed over the biodegradation of such man-made estrogenic chemicals [10]. The broad specificity for the phenolic substrates enables laccase to be developed as a biosensor for the determination of total phenols [11]. A fascinating character of the direct four-electron reduction of oxygen to water is another important application of laccase in the modified electrode of dioxygen sensing system [12] and in the cathode compartment of biofuel cells [13- 21].

Laccase is capable of oxidizing phenols by reducing molecular oxygen to water by a multicopper system. The catalytic center consists of three types of copper with different functions: type 1 (blue copper) catalyzes the electron transfer, type 2 activates molecular oxygen and type 3, a copper dimer, is responsible for the oxygen uptake [22].

The range of substrates which laccase can attack is very wide. In order to determine the catalytic process parameters in the present work the oxidation process of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) diammonium salt (ABTS) and 1,4-benzenediol (hydroquinone) were investigated with different techniques. Since laccases are increasingly being studied for practical reasons, knowing their activity as well as other catalytic reaction parameters becomes very important. An alternative electrochemical method for the determination of these values is proposed.

Experimental

Reagents and equipment:

Cerrena unicolor C-139 was obtained from the culture collection of the Regensburg University and deposited in the fungal collection of the Department of Biochemistry (Maria Curie-Sklodowska University, Poland) under the strain number 139. Laccase from the fermentor scale cultivation was obtained according to already reported procedure after ion exchange chromatography on DEAE-Sepharose (fast flow) [23] and lyophilized on Labconco (Kansas City, USA, FreeZone Lyophilizer). Enzyme activity was measured spectrophotometrically with syringaldazine as the substrate for laccase [24]. The protein content was determined according to Bradford with bovine albumine as the standard [25]. The concentration of isolated and freeze-d (-18°C) enzyme was $C_{\text{lacc}} = 178 \mu\text{g}\cdot\text{cm}^{-3}$ and activity $186,000 \text{ nkat}\cdot\text{dm}^{-3}$. After lyophilizing the laccase activity dissolved in 1 ml of water was $1,150,110 \text{ nkat}\cdot\text{dm}^{-3}$ and $C_{\text{lacc}} = 1.18 \text{ mg}/\text{cm}^3$.

2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonate (ABTS) was purchased from Sigma. Hydroquinone, Na_2HPO_4 , and citrate acid were from POCh. All reagents were used as received. Solutions were made up in Milli Q water ($18.2 \text{ M}\Omega\cdot\text{cm}^{-3}$).

The reaction rate was determined at substrates (ABTS and hydroquinone) concentration in the range of 0.01 to 1.5 mM in Mc Ilvaine buffer (0.1M citric acid + 0.2M NaH_2PO_4) at pH = 5.2, optimal for this laccase. All assays were performed in triplicate. The kinetic constant K_M for native laccase was calculated graphically using a Clark oxygen electrode (YSI Model 5300, Yellow Springs, Ohio, U.S.A) provided with a linear Pharmacia recorder REC 111 for hydroquinone [26] and Shimadzu model 160A UV-visible spectrophotometer for ABTS [27].

Voltammetry experiments were performed using the CHI 750B potentiostat (CH Instrument, Austin, USA) in a three-electrode arrangement, with a saturated Ag/AgCl electrode as the reference electrode and platinum foil as the counter electrode. The working electrodes were a platinum microelectrode with radius $r = \sqrt{2} \cdot 10^{-3} \text{ cm}$, glassy carbon electrode or gold disc electrode. Before each experiment, the electrodes were polished with aluminum oxide powder (grain size down to 0.05 μm) on a wet pad, rinsed with water and ethanol and then dried. The experiments were performed in McIlvaine buffer solution pH = 5.2, saturated with air. After dissolving 1.18 mg of laccase in 10 cm^3 of water, 300 μl of this solution was added to the solution of enzymatic substrate in buffer, at concentration in the range of 0.1mM – 1.5mM. Scans were recorded for 15 minutes one by one with the scan rate 0.04 V/s.

Methods:

Hydroquinone is often used as a laccase substrate in enzyme activity assays. The electrochemical behavior of this system is well known. Another advantage of using hydroquinone is its relatively good solubility in water, compared to that of other compounds, e.g., syringaldazine, used in laccase activity assays [3]. The molecular reaction models of oxidizing hydroquinone catalyzed with laccase given by Yaropolov et al. [28] can be simplified to the following equation:



The combination of blue copper oxidases (laccase, bilirubin oxidase), and ABTS as an electron transfer mediator, has been shown by Palmore and Kim [29] and

Ikeda and co-workers [30] to have a number of attractive features for oxygen-reducing fuel cell cathodes. Laccase oxidize ABTS according to equation (2):



Using cyclic voltammetry it is possible to monitor the dropping substrates or increasing products concentrations of enzymatic reaction, since the peak's currents are proportional to the concentrations. In the present paper microelectrodes working in the spherical diffusion zone are employed to follow the catalytic reaction. Measurements of stationary currents using microelectrodes are more convenient than measuring cyclic voltammetry peak heights – shape signals recorded using classical size electrode. Even application of differential pulse voltammetry does not allow obtaining well separated signals of the substrate and product with conventional size electrodes [Fig.1].

here Fig.1

The shape of the curves recorded at the microelectrode is wave-like, due to the different type of diffusion to the electrode surface [Fig.2A, B].

here Fig.2

Measuring the height of this wave is accurate and using microelectrodes allows miniaturization of the experimental system, which is especially convenient method for the determination of enzymatic activity.

The kinetic study was performed according to the Michaelis-Menten [31] theory, which allows analyzing quantitatively the enzymatic kinetics. The rate of the product formation at the steady state is characterized by V_0 and V_{max} : the initial and maximum rates, respectively, $[S]$ is starting substrate concentration and K_M , is the Michaelis-Menten constant. These constants were evaluated experimentally using the Lineweaver – Burk plot:

$$\frac{1}{V_0} = \left(\frac{K_M}{V_{\text{max}}} \right) \frac{1}{[S]} + \frac{1}{V_{\text{max}}} \quad (3)$$

$1/V_0$ was plotted against $1/[S]$, the slope of the line was K_M/V_{max} , the intercept was $1/V_{\text{max}}$, and the extrapolated $1/[S]$ intercept was $-1/K_M$.

Results

here Fig.3

Fig.3 represents the voltammograms recorded in the solution of ABTS (A) and hydroquinone (B) when the enzymatic reaction takes place. Increasing waves for the product and decreasing for the substrate of the enzymatic reaction are recorded in a time – scale of 30 to 900 sec. Measuring the limiting currents allows to follow both the disappearance of substrate and formation of product [Fig.4A,B].

here Fig.4

Since initial concentration of the substrate is known the values of the kinetic parameters were evaluated based on the changes of the substrate concentration. The reaction rate was determined using five different substrates (ABTS and hydroquinone) concentration in the range 0.01 to 1.5 mM [Fig.5A,B].

here Fig.5

After recalculating the limiting current values to the concentrations, the graphs of concentrations vs. time were plotted in their initial - linear range, where the slopes of the curves lead to the V_0 values. Based on the Lineweaver – Burk plots, the Michaelis-Menten constant and the maximal velocity were calculated [Fig.6].

here Fig.6

In case of ABTS, V_{\max} ($1/b$) is equal $219.44 \mu\text{mol}\cdot\text{min}^{-1}$, and K_M ($a\cdot V_{\max}$) is $459.29 \mu\text{mol}$. For the reaction when hydroquinone is the laccase substrate, $V_{\max} = 107.54 \mu\text{mol}\cdot\text{min}^{-1}$, and $K_M = 886.44 \mu\text{mol}$.

here Table 1

The values are collected in Table 1. The specific catalytic activity – catalytic activity divided by the mass of the protein – expressed as the number of enzymatic units per milligram of the protein is also given in Table 1. In order to compare the results obtained using the voltammetric method with those measured using other methods, the K_M was also determined using a Clark oxygen electrode for hydroquinone and spectrophotometric method for ABTS as the substrate. From the Lineweaver – Burk plots the K_M values were calculated. For hydroquinone as the substrate, the value is equal to $861.66 \mu\text{mol}$, which is in very good agreement with the value obtained using the voltammetric procedure (relative error $< 3\%$). In case of ABTS, the spectrophotometrically obtained K_M value is $418.6 \mu\text{mol}$, again in satisfied agreement with the result of the proposed electrochemical measurement (error of 8%), taking into consideration the complexity of material used. Specific catalytic activities are also compared. Values of activities obtained with our voltammetric approach using microelectrode and spectrophotometrically are close to each other (error of 6%);. The differences between values obtained using the microelectrode and Clark electrode methods are larger (error of 21%), but still acceptable.

Lately, Jarosz-Wilkolazka compared some fungal laccases and their specificity to different phenolic substrates [32]. The K_m values for *Cerrena unicolor* and ABTS as well hydroquinone obtained by her were much lower that probably comes from the composition of laccase preparation. The laccase used in her experiments was obtained from not optimized culture liquid and composed by two izoenzymes [33]. Laccase preparation used in this paper was performed after optimization of carbon, nitrogen and copper concentrations in the culture medium and was composed of four izoenzymes, each one specific to the different substrates [23]. The composition of this preparation gave much wider possibilities of a recognition of their potential laccase substrates.

Conclusions

The procedure for the measurement of the catalytic activity of laccase based on the application of voltammetry and microelectrodes working under spherical diffusion conditions are both accurate and simple. Very small volumes of solution and enzyme can be employed. The substrate concentration can be changed in a wide range. The voltammetric method proposed in this paper was compared with the two generally employed procedures based on oxygen measurement by Clark electrode and on spectrophotometry, and good agreement was obtained. Compared to spectrophotometry a larger number of compounds may be selected as substrates because colorless compounds can be used too. The procedure described in the present paper was found to be simpler and more reproducible results were obtained than the using Clark electrode. In addition, the voltammetric method allows to monitor parallelly the consumption of substrate and formation of product.

Acknowledgement

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FIGURE CAPTIONS

Fig.1 DPV voltammogram recorded at glassy carbon electrode in the solution containing hydroquinone and laccase in the buffer solution saturated with air. A – hydroquinone peak, C – quinone peak, arrows display the changes of signals with time, amplitude = 0.05V.

Fig.2 Diffusion model of species to the electrode surface: linear (A) and spherical (B) diffusion.

Fig.3 Voltammogram recorded at platinum microelectrode in the solution containing 1mM ABTS (A) or 1mM hydroquinone (B) and 0.035 mg laccase in the buffer solution saturated with air. A – oxidation wave, C – reduction wave, arrows display the changes of signals with time. Curves after 30 sec (a), 150 sec (b), 300 sec (c), 450 sec (d), 600 sec (e), 750 sec (f) and 900 sec (g); scan rate = 0.04V/s

Fig.4 Limiting currents of the oxidation and reduction waves versus time. Decreasing concentration of reduced form (solid line) and increasing of concentration of oxidized form (broken line) of ABTS (A) and hydroquinone (B) during catalytic reaction.

Fig.5 Current for (A) – ABTS, (B) – hydroquinone measured at the plateau of the oxidation wave vs. time during catalytic reaction. Initial concentrations: ■ 1.5mM, □ 1mM, ● 0.6mM, ○ 0.3mM, ▲ 0.1mM.

Fig.6 Lineweaver – Burk plot of laccase reaction with hydroquinone (solid line, $y=8.2426x+0.0093$, $R^2=0.9992$) and ABTS (broken line, $y=2.0926x+0.0046$, $R^2=0.9959$).

Table 1: Enzymatic activity of laccase. Comparison of catalytic reaction parameters obtained with different techniques.

METHOD	Clark electrode method		Spectrophotometric method		Voltammetric, microelectrode method	
	K_M [μmol]	specific catalytic activity [$\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$]	K_M [μmol]	specific catalytic activity [$\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$]	K_M [μmol]	specific catalytic activity [$\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$]
ABTS	-	-	418	58.35	455	62.00
Hydroquinone	861	38,54	-	-	886	30.38

Fig.1

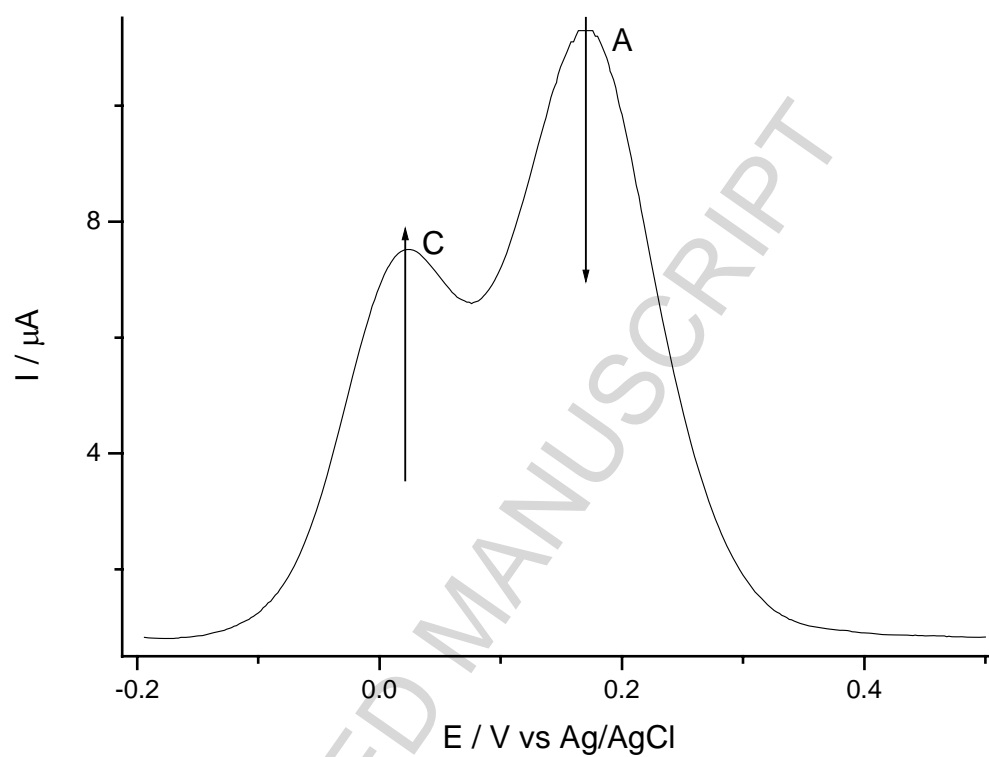
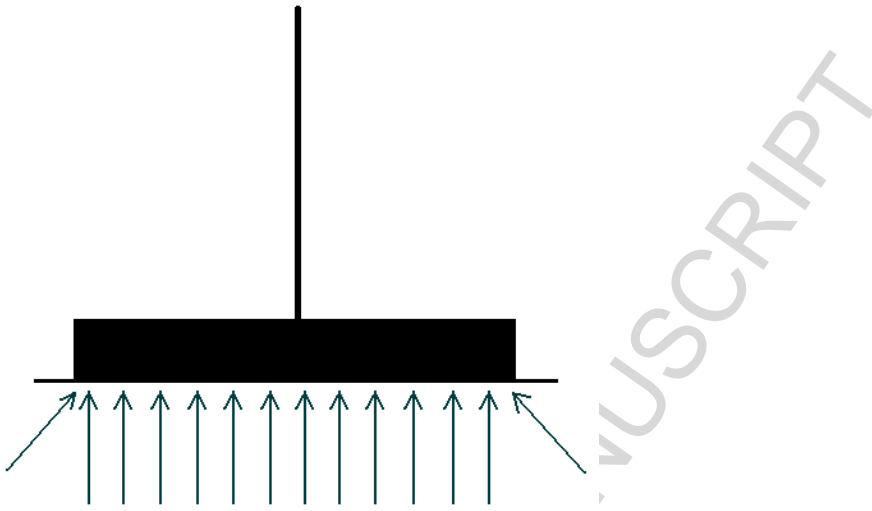
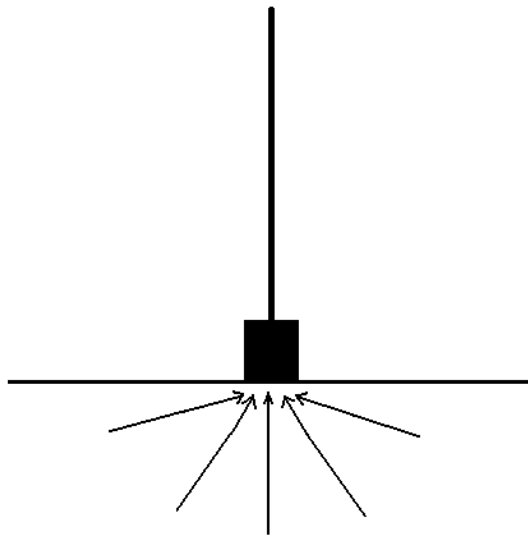


Fig.2

(A)

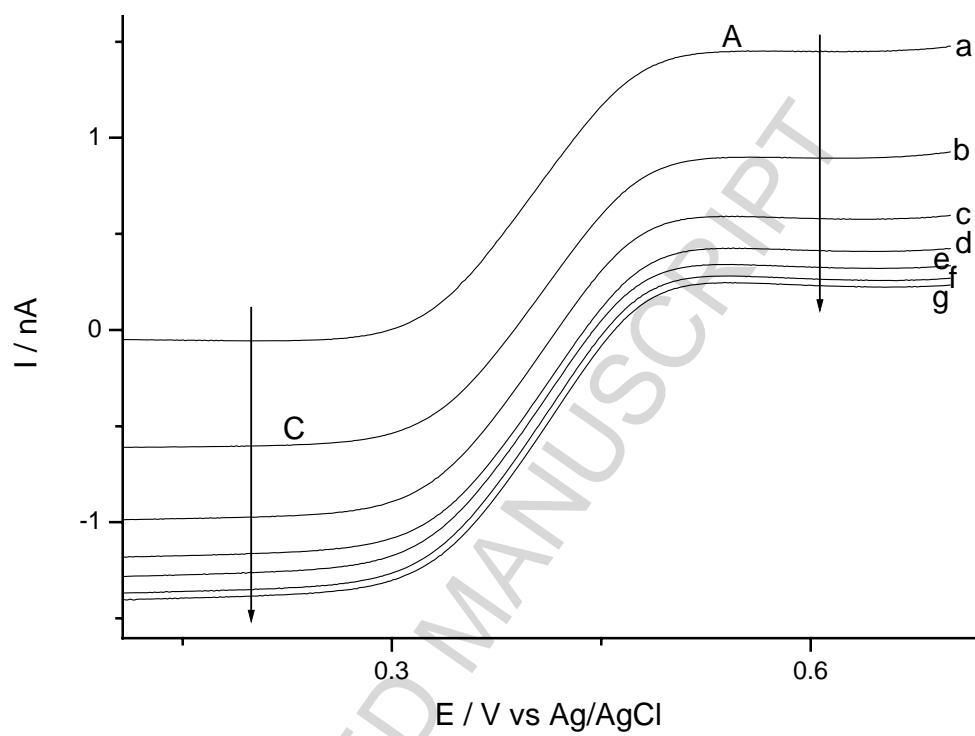


(B)



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Fig.3
(A)



(B)

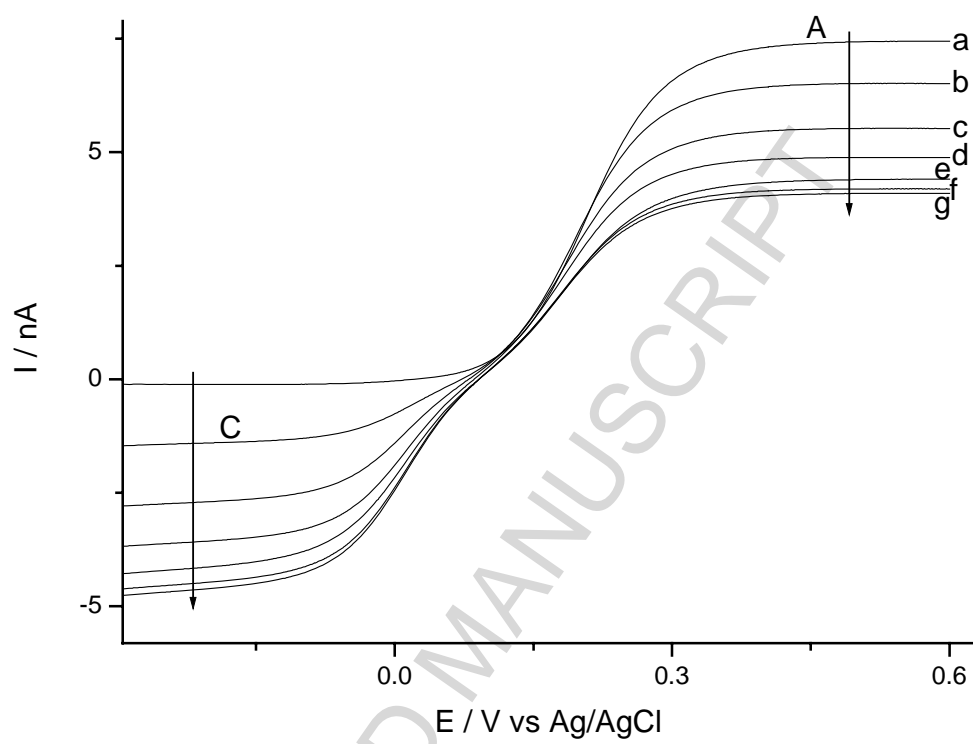
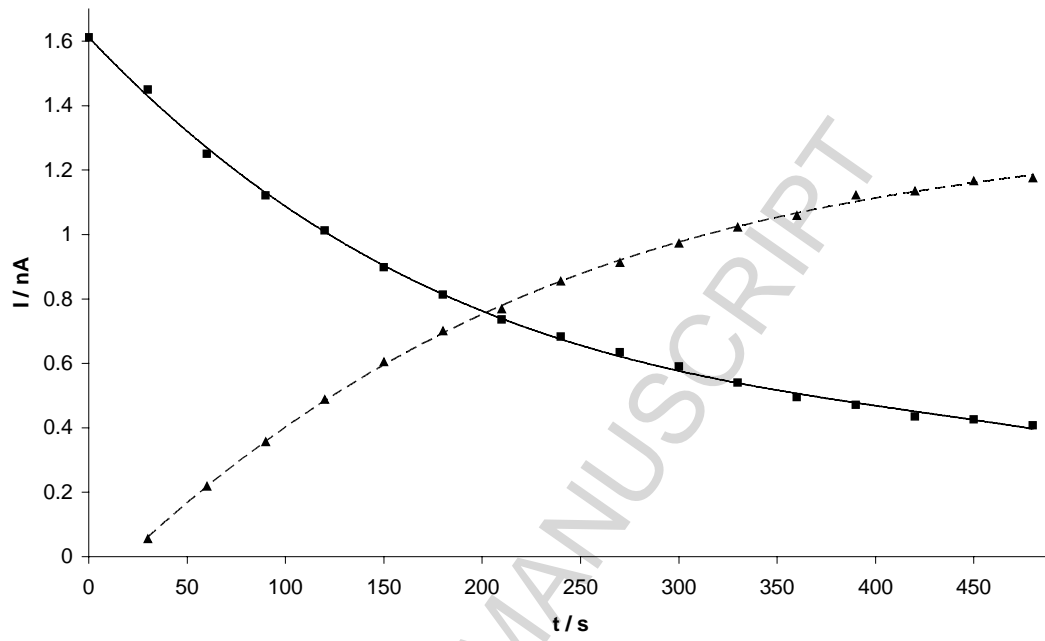


Fig.4
(A)

(B)

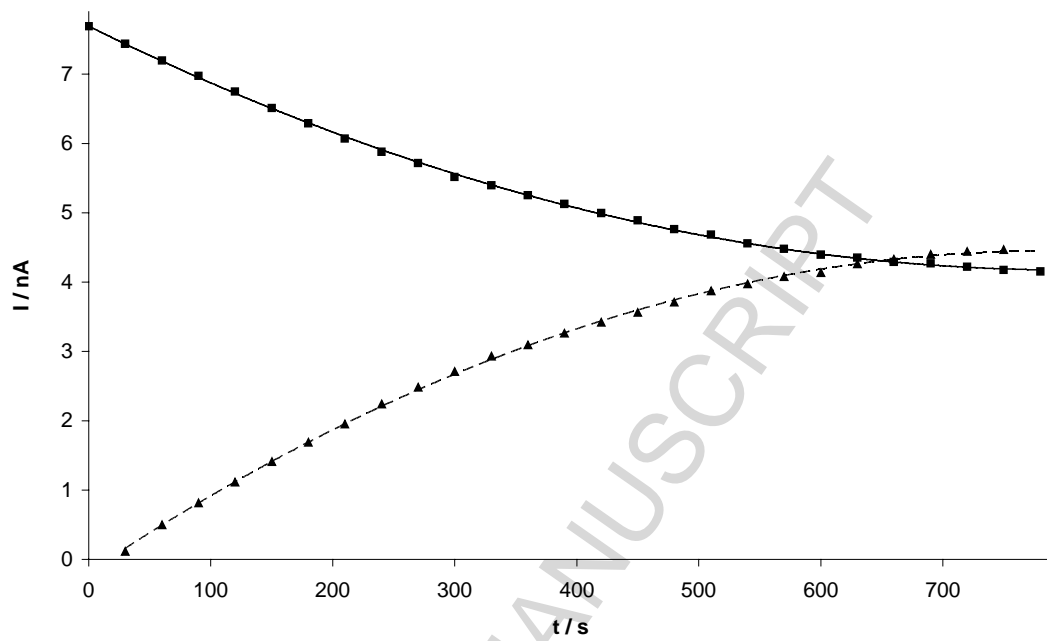
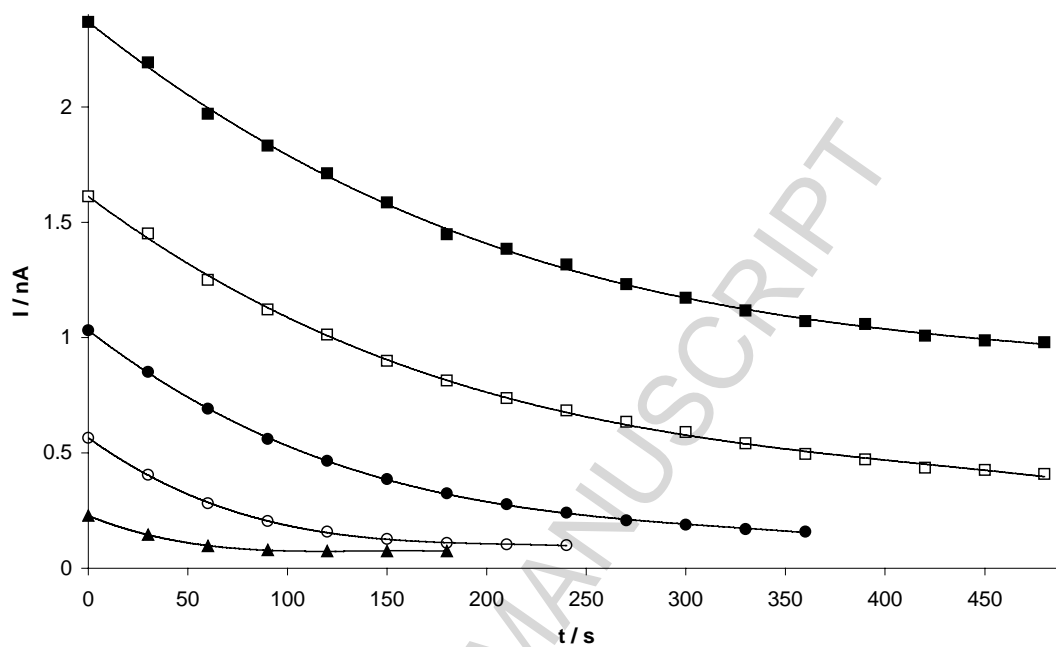


Fig.5
(A)



(B)

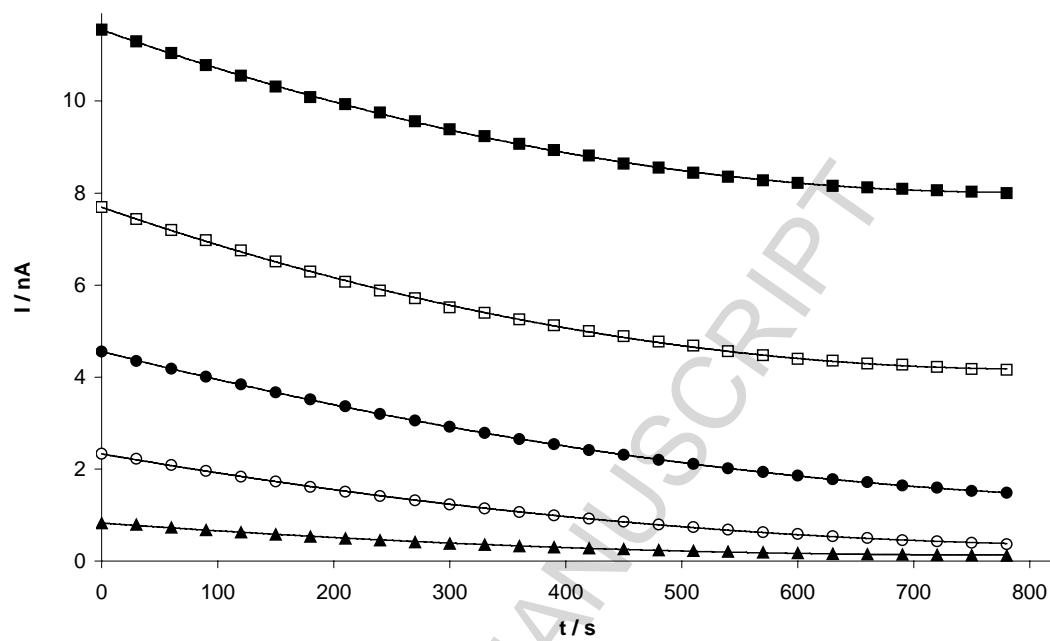


Fig.6

