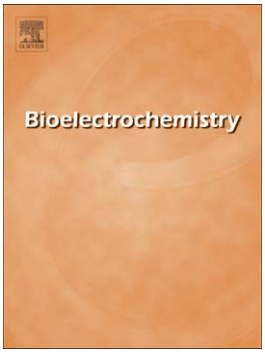

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PII: S1567-5394(14)00108-X
DOI: doi: 10.1016/j.bioelechem.2014.07.002
Reference: BIOJEC 6762

To appear in: *Bioelectrochemistry*

Received date: 24 July 2013
Revised date: 8 July 2014
Accepted date: 13 July 2014

Please cite this article as: Teodor Adrian Enache, Ana Maria Oliveira-Brett, Electrochemical evaluation of Glutathione S-transferase kinetic parameters, *Bioelectrochemistry* (2014), doi: 10.1016/j.bioelechem.2014.07.002

Electrochemical evaluation of Glutathione S-transferase kinetic parameters

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Abstract

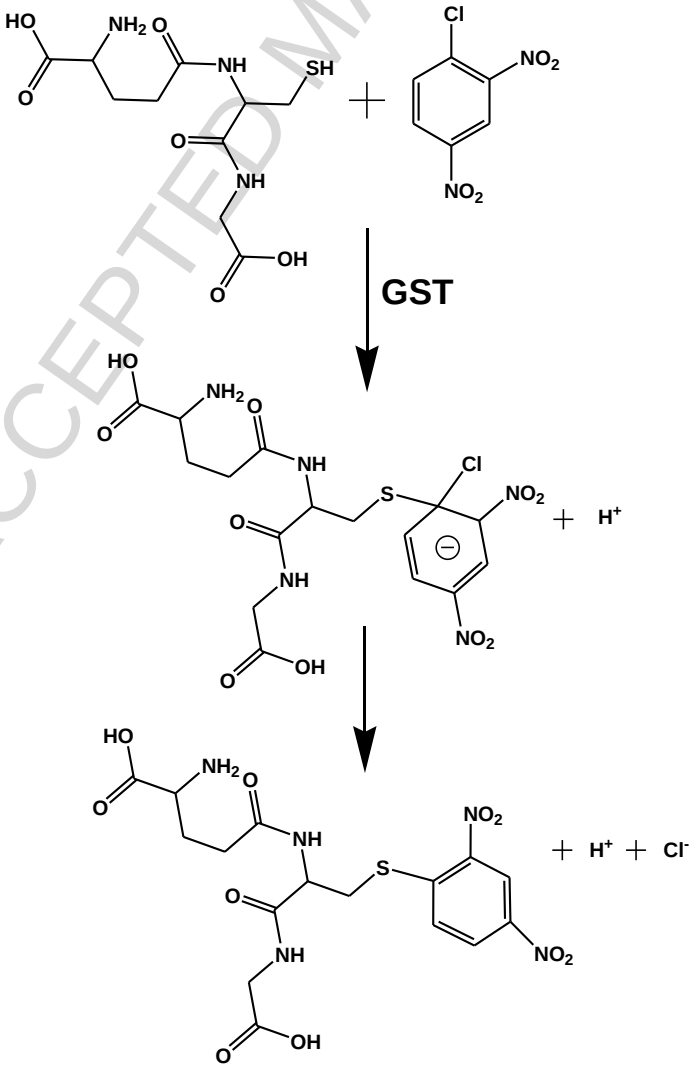
Glutathione S-transferases (GSTs), are a family of enzymes belonging to the phase II metabolism that catalyse the formation of thioether conjugates between the endogenous tripeptide glutathione and xenobiotic compounds. The voltammetric behaviour of glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione S-transferase (GST), as well as the catalytic conjugation reaction of GSH to CDNB by GST was investigated at room temperature, T = 298.15 K (25 °C), at pH 6.5, for low concentration of substrates and enzyme, using differential pulse (DP) voltammetry at a glassy carbon electrode. Only GSH can be oxidized, a sensitivity of 0.14 nA/µM and a LOD of 6.4 µM were obtained. The GST kinetic parameters electrochemical evaluation, in relation to its substrates, GSH and CDNB, using reciprocal Michaelis–Menten and Lineweaver–Burk double reciprocal plots, were determined. A value of K_M ~ 100 µM was obtained for either GSH or CDNB, and V_{max} varied between 40 - 60 µmol/min per mg of GST.

Keywords: glutathione, 1-chloro-2,4-dinitrobenzene, glutathione S-transferase, electrochemistry, oxidation

1 Introduction

The metabolism of cancer cells is controlled by oncogene signalling and by dysregulation of metabolic enzymes. The resulting altered metabolism supports cellular proliferation and survival but leaves cancer cells dependent on a continuous supply of nutrients. A primary cause of cancer treatment failure and patient relapse is an acquired or intrinsic resistance to anticancer therapies. Acquisition of drug resistance can be attributed to various factors that include avoidance of apoptotic cell death, altered expression of multidrug resistance-associated proteins, altered drug metabolism or uptake, and/or overexpression of phase II biotransformation enzymes [1, 2].

Many metabolic enzymes, such as those belonging to the phase II metabolism, have been investigated. Glutathione S-transferases (GSTs), one of the major phase II detoxification, are a family of enzymes that catalyses the formation of thioether conjugates between the endogenous tripeptide glutathione (GSH) and xenobiotic compounds, Scheme 1, [3]. They are abundant throughout most life forms [2], being involved in the metabolism of xenobiotics and play an important role in cellular protection against reactive and toxic electrophiles species that arise through normal metabolic processes [4].



Scheme 1 The conjugation reaction of GSH with CDNB by GST

From the structural point of view, two distinct superfamilies of GSTs have been described: the soluble cytosolic classes (Alpha, Mu, Pi, Kappa and Theta) and a microsomal family, designated as MAPEG (membrane-associated proteins in eicosanoid and glutathione metabolism) [5, 6], and the non- enzymatic functions of GSTs involve the interaction with proteins. Soluble GSTs and MAPEG are widely distributed throughout the body and are found in liver, kidney, brain, pancreas, testis, heart, lung, small intestine, skeletal muscles, prostate and spleen [3]

GSTs can catalyse a large number of reactions including nucleophilic aromatic substitutions, Michael additions, isomerizations and reduction of hydroperoxides, and play a major role in the detoxification of epoxides derived from polycyclic aromatic hydrocarbons and alpha-beta unsaturated ketone, quinones, sulfoxides, esters, peroxides and ozonides, and many endogenous compounds such as prostaglandins and steroids are also metabolized via glutathione conjugation reaction [3, 7, 8].

Specific substrates of GSTs have been already described [9]. Ethacrynic acid has been shown to be a very specific substrate for GST-P1 [10] and trans-stilbene oxide is a diagnostic substrate for GST-M1 [11]. Relatively small molecules, *e.g*. methylene chloride, ethylene dibromide or isoprene derivate have been shown to be conjugated by GST-T [12]. The 1-chloro-2,4-dinitrobenzene (CDNB) has been described as a universal GST substrate [13, 14], except for theta class enzymes which lack activity with this substrate [15].

Chemotherapeutic-resistant tumour cell lines have been shown to overexpress GST isozymes. This overexpression leads to an accelerated detoxification of drug substrates and thus an acquired resistance [1]. As a particular case, glutathione S-transferase Pi (GST-P) is a marker protein in many cancers (ovarian, non-small cell lung, breast, liver, pancreas, colon, and lymphomas) and high levels are linked to drug resistance, even when the selecting drug is not a substrate [16, 17]. Therefore, GSTs have emerged as a promising therapeutic target because specific isozymes are overexpressed in a wide variety of tumours and may play a role in the etiology of other diseases, including neurodegenerative diseases, multiple sclerosis, and asthma [17]. Consequently, there are many studies regarding GSTs substrate conjugation or inhibition reactions, most of them based on spectroscopic techniques, which require high concentrations, above millimolar, of protein and/or substrate [18-26].

However, no electrochemical assay for the determination of the kinetic parameters of GST was developed. The electrochemical techniques offer sensitivity and selectivity [27], making them very attractive tools for protein investigation [28, 29, 30]. Differential pulse voltammetry is recognised to be the most sensitive voltammetric method when the analyte is irreversible oxidized or reduced, and the glassy carbon electrode has excellent detection limits and high sensitivity, together being excellent tools for the redox behavior of biologic compounds investigation. In this research the catalytic conjugation reaction of CDNB and GSH catalysed by GST at micromolar concentrations, using differential pulse voltammetry and a glassy carbon electrode, was investigated. The results may contribute to an advance understanding of the enzymatic reactions occurring at low concentrations, which in turn can decrease the costs of new cancer research therapies.

2 Experimental

2.1 Materials and reagents

Reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione S-transferase (GST) from equine liver, from Sigma–Aldrich were used without further purification. Stock solutions of 1 mM GSH and CDNB (containing 30% (v/v) ethanol) were daily prepared in ultra-pure water from a Millipore Milli-Q system (conductivity ≤ 0.1 μS cm^{-1}). The GST solutions of different concentrations were prepared in 5% glycerol and were stored at -22 ºC.

The supporting electrolyte was 0.1 M phosphate buffer pH = 6.5.

Microvolumes were measured using EP-10 and EP-100 Plus Motorized Microliter Pippettes (Rainin Instrument Co. Inc., Woburn, USA). All experiments were done at room temperature (25 ± 1 ºC) in 0.1 M phosphate buffer pH =6.5.

2.2 Voltammetric parameters and electrochemical cells

Voltammetric experiments were carried out using a μAutolab running with GPES 4.9 software, Metrohm/Autolab, Utrecht, The Netherlands. Measurements were carried out using a glassy carbon electrode (GCE) as working electrodes, a Pt wire as counter electrode and an Ag/AgCl (3 M KCl) as reference electrode. The experimental conditions for differential pulse (DP) voltammetry were: pulse amplitude 50 mV, pulse width 70 ms and scan rate 5 mV s^{-1}.

The GCE (d = 1 mm) was polished using diamond spray (particle size 3 μm) before each experiment. After polishing, the electrode was rinsed thoroughly with Milli-Q water for 30 s; then it was placed in supporting electrolyte and various DP voltammograms were recorded until a steady state baseline voltammogram was obtained.

2.3 Acquisition and presentation of voltammetric data

All the voltammograms presented were background-subtracted and baseline-corrected using the moving average with a step window of 3 mV included in GPES version 4.9 software. This mathematical treatment improves the visualization and identification of peaks over the baseline without introducing any artefact, although the peak height is in some cases reduced (<10%) relative to that of the untreated curve. Nevertheless, this mathematical treatment of the original voltammograms was used in the presentation of all experimental voltammograms for a better and clearer identification of the peaks. The values for peak current presented in all graphs were determined from the original untreated voltammograms after subtraction of the baseline.

3 Results and discussion

An electrochemical method for evaluation of GST activity and determination of its kinetic parameters was developed. The conjugation reaction of GSH with CDNB catalysed by GST was studied by DP voltammetry in solutions incubated for different time periods and different concentrations of enzyme and substrates in 0.1 M phosphate buffer pH = 6.5.

GSH oxidation occurs at the cysteine residue and is an irreversible, diffusion-controlled, pH dependent process that involves the sulfhydryl group oxidation [28, 31].

GST catalyses the proton removal from GSH to generate the thiolate anion GS^-, that is more reactive than GSH. The thiolate conjugation reaction with CDNB occurs at carbon one where chloride was bound, producing a Meisenheimer complex. This complex is unstable, chloride dissociates, and the glutathionyl-dinitrobenzene (GS-DNB) conjugate is formed in solution [32], Scheme 1.

Therefore, as GST catalyses the conjugation reaction of CDNB, less free GSH oxidizable sulfhydryl groups are be available in solution to react. Consequently, the formation of the GS-DNB complex as well as the GST activity can be indirectly determined by the electrochemical evaluation of the GSH oxidation current decrease.

The DP voltammograms were recorded using a clean GCE surface and the current corresponding to GSH sulfhydryl group oxidation was measured in order to quantify the GS-DNB product. The CDNB, GST, and conjugation reaction product GS-DNB, were not electroactive in the experimental conditions used.

3.1 GSH electrochemical oxidation

GSH electrochemical oxidation occurs at the sulfhydryl groups. DP voltammetry at a clean GCE surface, in [GSH] = 50 µM, in 0.1 M phosphate buffer pH = 6.5, showed one oxidation peak, at E_p = 0.54 V, with peak current I_p ~ 8 nA, Fig.1.

Here Fig.1

The electroanalytical determination for standard additions of GSH, final bulk concentrations between 5 - 150 µM, gave a LOD of 6.4 µM and a LOQ of 21.5 µM. The data extracted from the calibration curve showed, by the value of R^2 = 0.997, a wider linear range between 5 and 120 µM following the equation $y = b[x] + a$, where y represents the current in nA, b = 0.145 nA/µM is the sensitivity, [x] in µM is the GSH concentration and a = 0.87 nA represents the OY intercept. The relative standard deviation (R.S.D.), calculated from three calibration curves was less than 7%. This error derives from the fact that each measurement was always done using a newly polished GCE surface, a process that gives rise to small changes in the electrode surface area, which can in turn cause small variations in the currents measured. The GSH detection limit (LOD) was determined from the equation $LOD = 3 \times SD \times (\text{sensitivity})^{-1}$, where SD is the standard deviation of the response, and the quantification limit (LOQ), the lowest concentration that can be quantified with acceptable precision and accuracy, as $LOQ = 10 \times SD \times (\text{sensitivity})^{-1}$.

The influence of GST and CDNB on the oxidation peak current of GSH was investigated. A solution containing [GSH] = 50 µM was incubated for 1 h with [CDNB] = 50 µM or mass of enzyme GST (m(GST)) = 50 ng, and no significant changes were observed to the GSH peak potential or current in the presence of CDNB or GST, Fig. 1.

The non-enzymatic reaction between GSH and CDNB [24, 32] takes place with a low reaction rate at very high concentrations. The formation of GS-DNB was not detected for the lower micromolar concentration used. After 12 h incubation time of [GSH] = 50 µM with [CDNB] = 50 µM and m(GST) = 50 ng, the GSH oxidation peak completely disappeared, Fig. 1.

3.2 GST activity electrochemical evaluation

The indirect determination of the GS-DNB complex and GST activity was electrochemically evaluated based on the decrease of the GSH sulfhydryl group oxidation peak current.

The effect of varying m(GST) on the conjugation reaction rate in solutions containing [GSH] = 50 µM and [CDNB] = 50 µM was investigated for four incubation periods, 0, 15, 30 and 60 min.

At 0 min, no significant differences were observed on the GSH oxidation peak current, even in a GST concentrated solution, Fig. 2. Increasing the incubation time and GST concentration, the GSH oxidation peak current decreased slowly for a small concentration of enzyme and faster in GST concentrated solutions, Fig. 2.

Here Fig. 2

The DP voltammograms in solution containing [GSH] = 50 µM and [CDNB] = 50 µM incubated for 15 and 30 min and for a varying m(GST) concentration, between 6 - 150 ng, showed a decrease of the GSH oxidation peak current I_p with increasing enzyme concentration, Fig. 3A.

Here Fig. 3

The enzyme activity are the number of moles of substrate converted per unit time or the rate of a reaction times the reaction volume, and the units used are one enzyme unit (U) equal to 1 µmol min^{-1}. The rate of a reaction is determined by the concentration of substrate disappearing (or product produced) per unit time, and the rate of a reaction units are mol L^{-1} s^{-1}.

The initial current I_i, measured before enzymatic reaction, *i.e.* before incubation, corresponds to a given initial $[GSH]_i$ concentration, and follows the linear relationship I(nA) = 0.145 [*GST*] + 0.87. The final current I_f is obtained after incubation and is related to the remaining unconjugated $[GSH]_f$ concentration in solution.

The difference between initial $[GSH]_i$ and unconjugated $[GSH]_f$ corresponds to the [GS-DNB] formed, and $I_i - I_f$ is the decrease in current due to the conjugated [*GSH*] formation. Therefore, the molar concentration of conjugation reaction product [GS-DNB], for each experimental condition can be calculated as:

$$[GS-DNB] = \frac{[GSH]_i (I_i - I_f)}{I_i} \qquad \text{Eq. 1}$$

The enzyme activity, Eq.2, describes the µmol of GSH that reacted with CDNB per minute. The $[GSH]_i$ = 50 µM used is within the linear calibration curve region, where the current is directly proportional to concentration. The GSH concentration conjugated to CDNB corresponds to the difference between the initial oxidation peak current (I_i), obtained for [GSH] = 50 µM before incubation, and the [GSH] final oxidation peak current (I_f), measured after incubation (15 or 30 min), in 200 µL, 0.1 M phosphate buffer pH=6.5. The *Enzyme Activity* = $([GSH]_i \cdot (I_i - I_f) \cdot V_r)/I_i \cdot t$, Eq. 2. Where the enzyme activity is in U (enzyme units); 1U = 1 µmol/min; $[GSH]_i$ = 50 µM (50 µmol/L) is the GSH initial concentration; I_i = 8.3 nA is the oxidation peak current for $[c_i]$ = 50 µM before incubation; I_f is the oxidation peak current of unconjugated $[c_i]$ after incubation; $V_r = 200 \times 10^{-6}$ L is the reaction volume; *t* is the incubation time in minutes (15 or 30 min).

The enzyme activity increases linearly with the enzyme concentration up to ~50 ng, where the reaction starts to be limited by substrate concentration, Fig. 3B, and the reaction rate becomes constant. The enzyme activity calculated for 30 min incubation time was lower than the enzyme activity calculated for 15 min incubation time, due to the decrease of substrates concentration, Fig. 3B.

3.3 GST determination of K_M and V_{max} values

The GST kinetic parameters, for different concentrations of GSH and CDNB incubated for two time periods (15 and 30 min) with m(GST) = 13 x 10^{-6} mg, in 200 µL, in 01M phosphate buffer pH=6.5, were determine, Figs. 4 and 5. GST is a two substrate enzyme, which catalysis the formation of a thioether conjugate between the endogenous tripeptide glutathione (GSH) and a xenobiotic compound, here CDNB, and only the consumption of GSH was detected. After incubation, the DP voltammograms were recorded at a clean GCE surface and the GSH oxidation peak current was used for the indirect quantification of GS-DNB complex formed.

The Michaelis–Menten equation model was derived to account for the kinetic properties of enzymes. The kinetic parameters – the Michaelis constant (K_M) and the maximal reaction velocity (V_{max}) – were determined by two methods: Lineweaver–Burk (double reciprocal) transformation, Figs. 4A and 5A, and nonlinear curve-fiting of Michaelis–Menten reciprocal plot, Figs. 4B and 5B.

Here Fig. 4

Here Fig. 5

The GST activity was investigated for concentrations varying between 20-150 µM for CDNB or GSH. The concentration of the one substrate, [CDNB] or [GSH], was kept constant at 100 µM. The values of the K_M and V_{max} of GST for substrates CDNB and GSH, were determined, Table 1.

Here Table 1

The GST initial reaction velocities for GSH ($V_{i\text{-}GSH}$), equation (**3**), and CDNB ($V_{i\text{-}CDNB}$), equation (**4**), were calculated as µmol of GSH conjugated to CDNB per minute per mg m(GST). Considering the [GSH] calibration curve, the conjugated [GSH] was calculated as $[GSH]_i$-$[GSH]_f$, and the conjugated [GSH] current is $I_i - I_f$, with m(GST) = 13x10^{-6} mg, in 200 µL, in 0.1 µM phosphate buffer pH=6.5.

Therefore, $V_{i\text{-}GSH} = ([GSH]_i \cdot (I_i - I_f) \cdot V_r)/I_i \cdot t \cdot m(GST)$, Eq. 3, where: $[GSH]_i$ = 20-150 µM (20-150 µmol/L) is the initial GSH concentration; I_i is the initial current obtained for $[c_i]$ before incubation; and $V_{i\text{-}CDNB} = ([GSH]_i \cdot (I_i - I_f) \cdot V_r)/I_i \cdot t \cdot m(GST)$, Eq. 4, where: $[GSH]_i$ = 100 µM (100 µmol/L) is the initial GSH concentration; I_i = 14 nA is the initial current obtained for $[c_i]$ before incubation; in both Eq. 3 and 4, $V_{i\text{-}GSH}$ is the initial velocity in µmol min^{-1} mg^{-1}; m(GST) = 13x10^{-6} mg is the enzyme amount; I_f is the final current obtained for remaining $[c_i]$ after incubation; V_r = 200 x 10^{-6} L is the reaction volume; t is the incubation time in minutes (15 or 30 min).

The Lineweaver–Burk plot is a classic method but as the Y-axis takes the reciprocal of the V_i any small errors in the measurements will be increased. Also, when experimental conditions do not allow large concentrations of substrate, *e.g*. saturation or low solubility, there will be no small values for 1/[S], which will give a large intercept

extrapolation value [33]. The nonlinear curve-fitting of Michaelis–Menten reciprocal plot, Fig.4, ensure an accuracy value of V_{max} since the fit Eq. 5:

$$V_i = \frac{V_{max}[S]}{K_M + [S]} \quad \text{Eq.5}$$

The results obtained for different concentrations of [GSH] and [CDNB] incubated for 15 and 30 min, Figs. 4 and 5, showed slightly different K_M values varying between 96-111 µM, whereas for short incubation times V_{max} highest values were obtained, Table 1. The statistical analysis revealed that GST showed the same affinity to the either substrate CDNB or GSH. Nevertheless, the Lineweaver–Burk plots indicated that both GSH and CDNB are GST uncompetitive substrates.

The most common method employed for the evaluation of GST kinetic parameters is spectroscopy [18-26, 32]. Usually, depending on the GST isozyme type and experimental assay, K_M varies between 0.1 mM [25] and more than 1 mM [26]. However, spectroscopic methods request large quantities of analytes, 0.1 – 5 mM for substrates and more than 10 µg of enzyme, in order to ensure a reasonable time for each assay. On the other hand, in agreement with the lowest values reported [25], due to the high sensitivity of DP voltammetry was possible to use a low GST concentration, and a low K_M was determined.

4 Conclusions

A rapid, efficient and sensitive electrochemical method for the determination of the kinetic Michaelis constants of glutathione S-transferase activity was developed measuring the GSH sulfhydryl group's oxidation peak current at GCE by DP voltammetry. The GSH detection limit was 6.4 µM and quantification limit was 21.5 µM. The effect of enzyme and substrates concentration on the enzymatic reaction rate, as well as the influence of GST and CDNB on the GSH oxidation peak current, was investigated. The optimum experimental conditions were low enzyme concentration, below 250 ng/mL (50 ng in 200µL reaction volume), and 15 min incubation time. The K_M ~ 100 µM for either GSH or CDNB was obtained, showing the same affinity of GST for both substrates.

The sensitivity of the electrochemical methodologies has the advantage of enabling low detection limits which means low reagent consumption and can contribute to a diminution of the total costs associated with cancer therapy research. The use of screen printed electrodes is foreseen, thus enhancing the applicability of electrochemical methodologies for the determination of the kinetic Michaelis constants with a miniaturised and portable device.

5 Acknowledgements

Financial support from Fundação para a Ciência e Tecnologia (FCT), Post-Doctoral Grant SFRH/BPD/80195/2011 (T.A. Enache), projects PTDC/QEQ-MED/0586/2012, PEst-C/EME/UI0285/2013 and CENTRO-07-0224-FEDER-002001 (MT4MOBI) (co-financed by the European Community Fund FEDER), FEDER funds through the program COMPETE–Programa Operacional Factores de Competitividade, is gratefully acknowledged.

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Table Captions

Table 1. GST kinetic parameters electrochemical evaluation

Figure Captions

Fig. 1 DP voltammograms baseline corrected in 0.1 M phosphate buffer pH = 6.5 after 1 hour incubation period: (**—**) 50 µM GSH, (**- - -**) 50 µM GSH + 50 ng GST, and (**•••**) 50 µM GSH + 50 µM CDNB; and after 12 hours incubation period (**-•-•**) in 50 µM GSH + 50 µM CDNB + 50 ng GST.

Fig. 2 3D plots of DP voltammograms in 50 µM GSH + 50 µM CDNB with different GST concentration for different incubation periods.

Fig. 3 Variation of GSH: (A) I_p oxidation peak current and (B) enzyme activity for (○) 15 and (■) 30 min incubation time of [GSH] = 50 µM with [CDNB] = 50 µM, and m(GST) varying between 6 - 150 ng; 1 U = 1 µmol min^{-1}; The dotted lines represent the data fit.

Fig. 4 Plots after 15 min incubation period: (■) GSH and (○) CDNB: (A) Lineweaver–Burk double reciplocal and (B) Michaelis–Menten reciprocal. The dotted lines represent the data fit.

Fig. 5 Plots after 30 min incubation period: (■) GSH and (○) CDNB: (A) Lineweaver–Burk double reciprocal and (B) Michaelis–Menten reciprocal. The dotted lines represent the data fit.

Table 1. GST kinetic parameters electrochemical evaluation

METHOD	GSH				CDNB			
	K_M (µM)		V_{max} (µmol/min mg)		K_M (µM)		V_{max} (µmol/min mg)	
	15 min	30 min	15 min	30 min	15 min	30 min	15 min	30 min
Nonlinear fit	100	104	58	40	100	104	59	49
Lineweaver–Burk	104	107	58	43	111	107	62	48

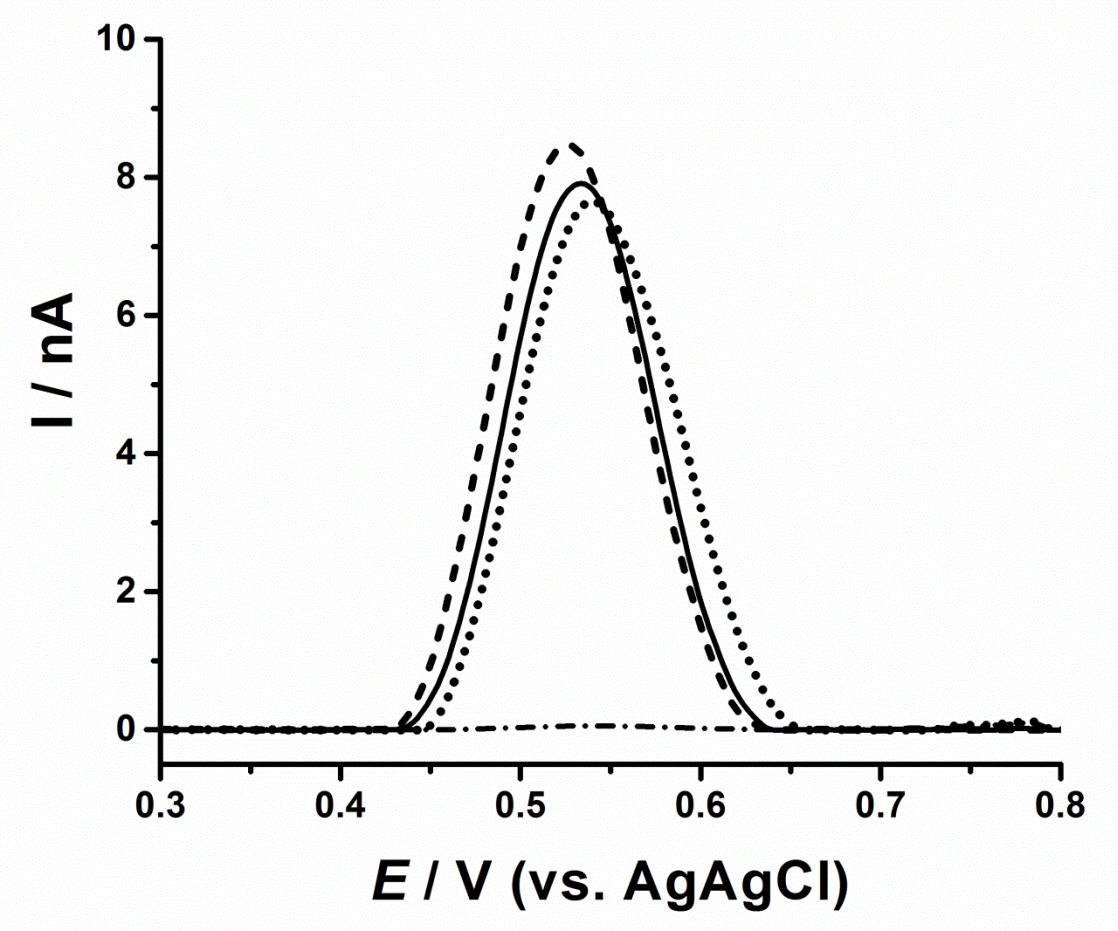

Fig. 1

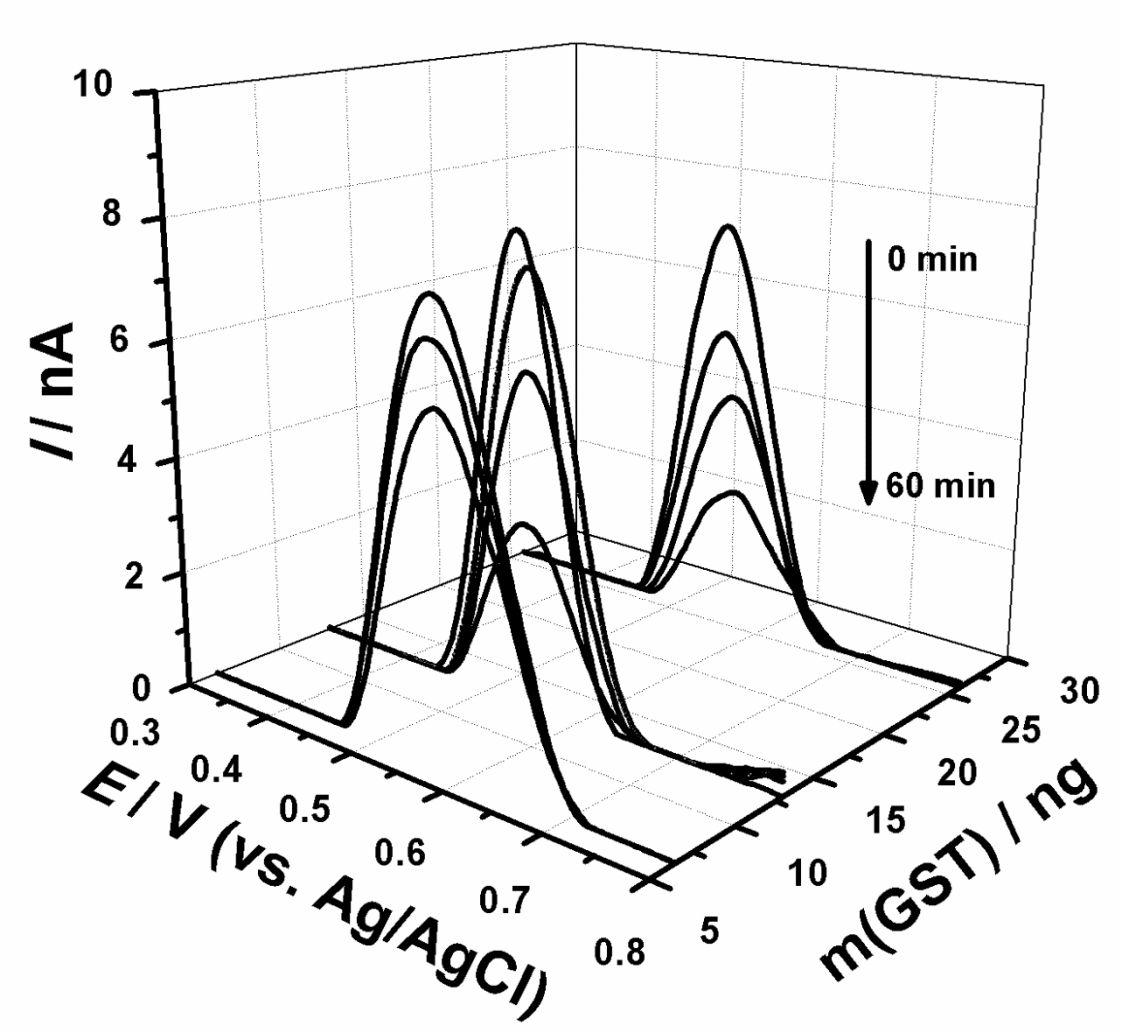

Fig. 2

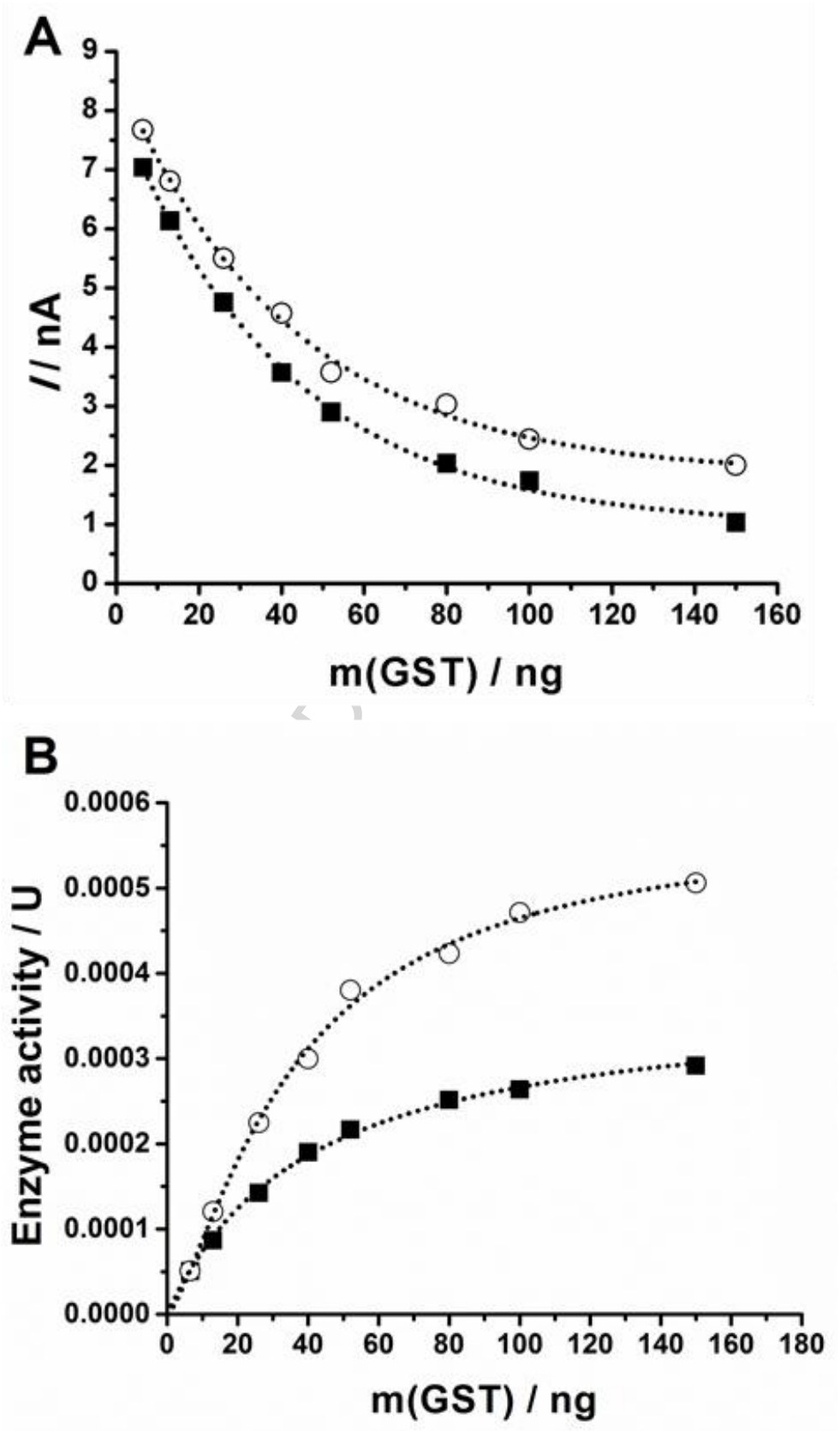

Fig. 3

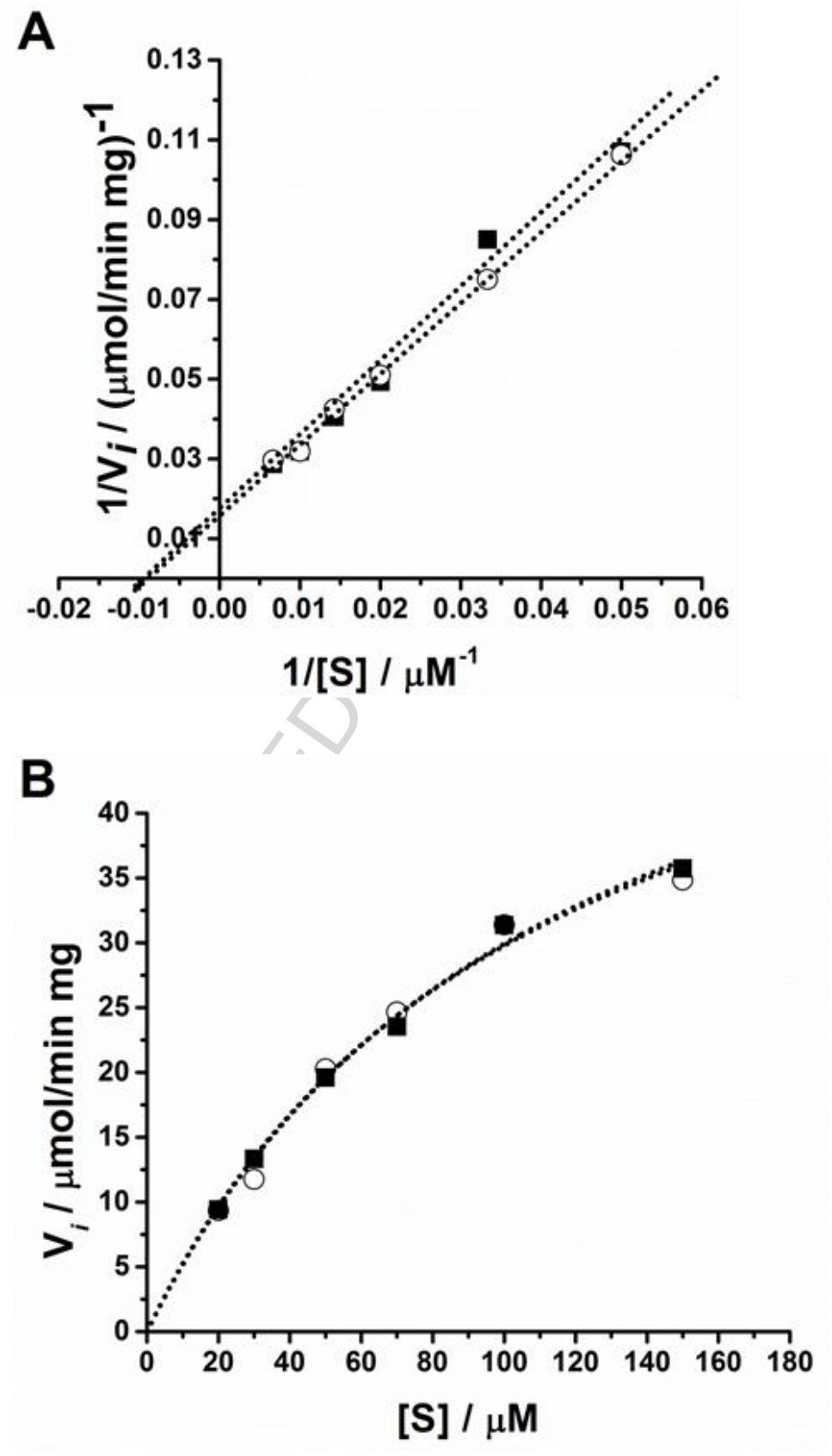

Fig. 4

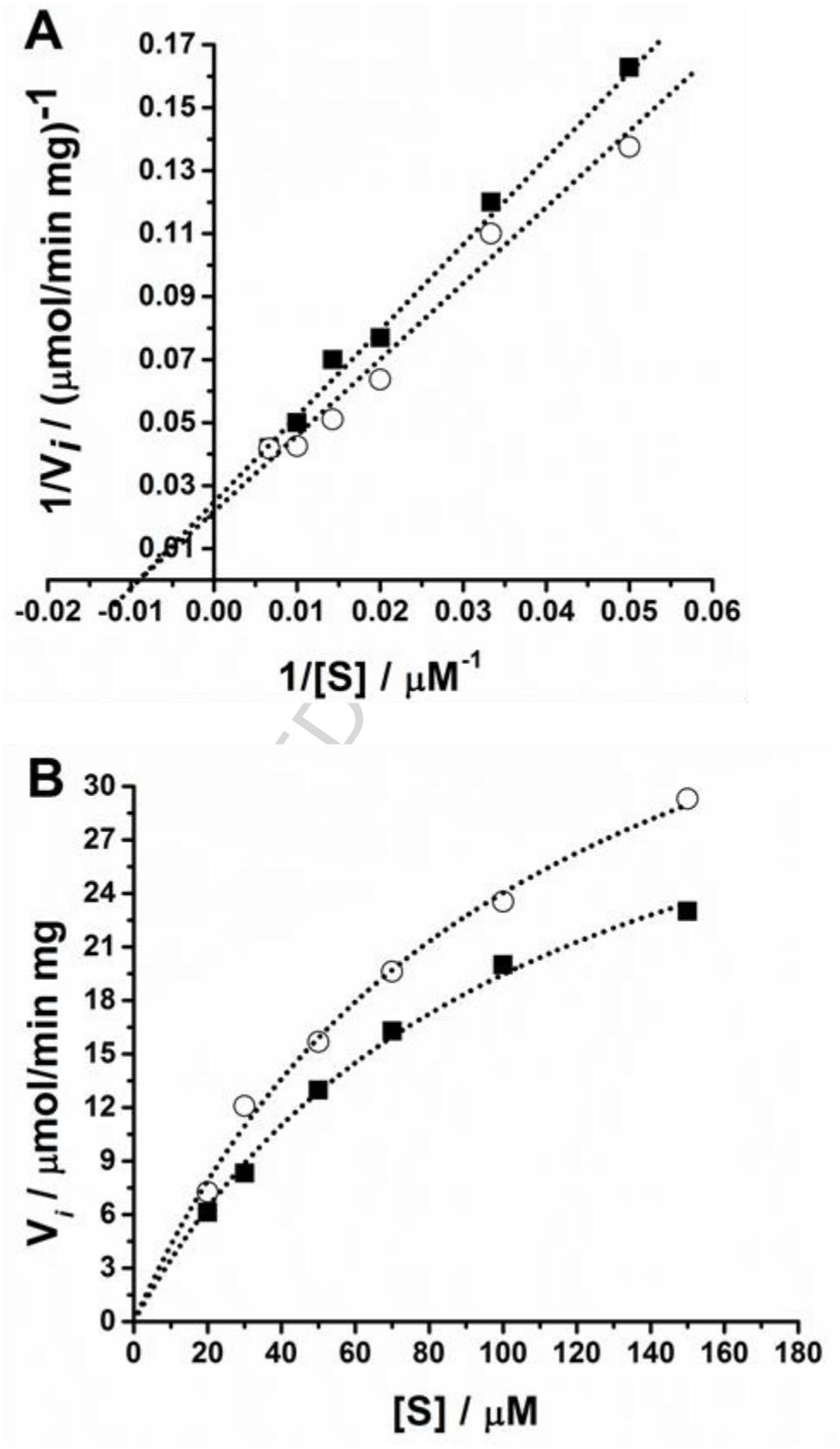

Fig. 5

Electrochemical evaluation of Glutathione S-transferase kinetic parameters

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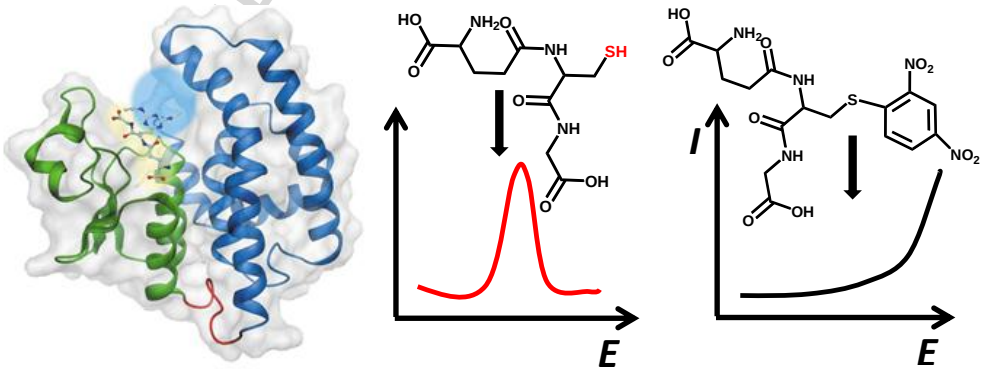
Graphical Abstract



Electrochemical evaluation of Glutathione S-transferase kinetic parameters

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Highlights

- This work deals with GST kinetic parameters evaluation
- GSH as endogenous and CDNB as xenobiotic substrates, were used
- Electrochemical detection of the kinetic parameters at low enzyme concentration
- Michaelis–Menten and Lineweaver–Burk plots gave a K_M ~ 100 μM for either GSH or CDNB