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Electrochemical study of *S*–nitrosoglutathione and nitric oxide by carbon fibre NO sensor and cyclic voltammetry – possible way of monitoring of nitric oxide

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Abstract

Nitrosation of sulfhydryl group of glutathione, which is highly reactive and is often found conjugated to other molecules via its sulfhydryl moiety, is one of many biological effects of the nitric oxide (NO). This process may serve as a signal event and/or as a deposition of NO to S–nitrosoglutathione (GSNO). Moreover, GSNO may release NO under specific conditions. That is why NO, which has a little lifetime itself, could be distribute for longer distances within the organism. Here, we studied and compared the basic electrochemical characteristics of biological active thiol compounds (GSH, oxidized glutathione and GSNO). In addition, observation of the decomposition process of GSNO using different electrochemical techniques followed. Primarily we studied the influence of scan rate and reducing agent (Tris(2-carboxyethyl)phosphine). The CV calibration equations were linear, R.S.D. about 5%. The detection limits of GSH, GSSG and GSNO expressed as 3 S/N were 9 nM, 4 nM and 20 nM, respectively. In addition, the use of NO selective carbon fibre electrode and cyclic voltammetry for the study of GSNO decomposition catalysed by copper(II) and iron(II) followed.

Keywords: Glutathione; GSH; GSSG; Nitrosoglutathione; *S*-nitroso-*N*-acety-*D*,*L*-penicillamine; Thiols; Nitric oxide; Cyclic voltammetry; Carbon fibre electrode; Copper; Iron

1. Introduction

Glutathione (GSH, Fig. 1A) was discovered by F. G. Hopkins in 1921 [1]. It is a ubiquitous tripeptide containing sulfhydryl group and attracts great deal of attention of many scientists [2–4]. Its major functions are concerned to redox balance maintenance, redox signalling and detoxification of heavy metals and some xenobiotics [5–10]. Besides reduced form of GSH, oxidized form called GSSG (Table 1A) is also very important molecule, because changes in the ratio of intracellular reduced and disulfide forms of glutathione (GSH/GSSG) can affect signalling pathways that participate in various physiological responses from cell proliferation to gene expression and apoptosis [11,12].

Recently, it was discovered that GSH is connected to the nitric oxide (NO) metabolism [13–15]. NO is one of the most important signalling molecules in animals and even in plants [16–18]. Nitrosation of sulfhydryl group of glutathione, which is highly reactive and is often found conjugated to other molecules via its sulfhydryl moiety [2], is one of many biological effects of the NO [19]. This process may serve as a signal event and/or as a deposition of NO to S–nitrosoglutathione (GSNO, Fig. 1A) [20]. Moreover, GSNO may release NO under specific conditions [20–22]. That is why NO, which has a little lifetime itself, could be distribute for longer distances within the organism [20,23].

Various flow electrochemical techniques for the determination of thiols such as cysteine, glutathione, metallothionein have

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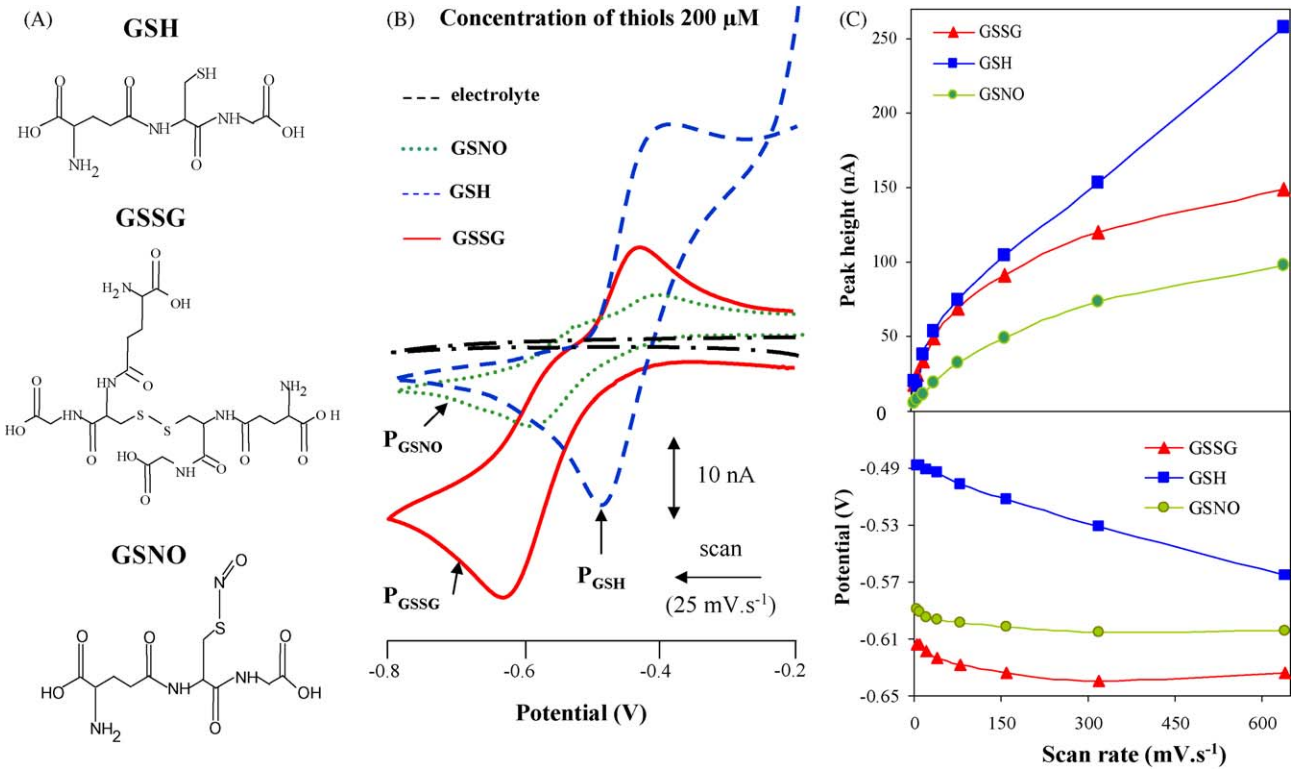

Fig. 1. Electrochemical behaviour of reduced (GSH) and oxidized glutathione (GSSG), and *S*-nitrosoglutathione (GSNO). (A) Chemical formulas of GSH, GSSG and GSNO. (B) Typical cyclic voltammograms of the studied thiols (200 μM) – GSH (broken line), GSSG – without TCEP (continuous line), GSNO – without TCEP (dotted line) and background electrolyte (0.05 M $Na_2B_4O_7$, pH 9.2 with 1 mM Tris(2-carboxyethyl)phosphine – TCEP; dash-and-dot line). The arrows are showing cathodic peaks of GSH (P_{GSH}), GSSG (P_{GSSG}) and GSNO (P_{GSNO}). (C) Dependence of peak height and peak potential on scan rate. CV parameters were as follows: scan rate 25 mV s^{-1} (except Figure C), step potential 5 mV, start potential −0.2 V, vertex potential −0.8 V, deoxygenating by argon for 140 s. Background current did not change much with increasing scan rate (about 10%). For other details see Experimental section.

been reported, such as liquid chromatography (LC) [24–30] and/or capillary electrophoresis (CE) [31–35]. Moreover, a number of stationary electrochemical techniques including voltammetry [36–39] and chronopotentiometry [40–44] have been suggested too. On the other hand, a few authors describe the electrochemical behaviour of GSNO [45–49].

In our previous work we utilized cyclic voltammetry (CV) in combination with hanging mercury drop electrode (HMDE) to determine GSH and GSSG. This method does not require any derivatization reagent and reduced and oxidized glutathione may be detected simultaneously [50]. On the base of these results, here we studied and compared the basic electrochemical characteristics of biological active thiol compounds (GSH, oxidized glutathione and GSNO). In addition, observation of the decomposition process of GSNO using different electrochemical techniques followed.

2. Materials and methods

2.1. Chemicals

Reduced glutathione (GSH), oxidized glutathione (GSSG) and *S*-nitrosoglutathione (GSNO) were purchased from Sigma–Aldrich (St. Louis, USA). Tris(2-carboxyethyl)phosphine

Table 1
Determination of thiols (reduced and oxidized glutathione, and *S*-nitrosoglutathione) by cyclic voltammetry ($n = 10$)

Thiols	Peak potential (V)[a]	Concentration of thiols[b]	Regression equation[c]	R^2	L.O.D.[d] (nM)
GSH	−0.49	10–190[1]	$y = 0.1938x - 0.7428$[a]	0.9988	9
		50–1000[2]	$y = 0.2303 + 2.536$[b]	0.9974	
GSSG	−0.62	10–190[1]	$y = 0.1735x + 1.4415$[a]	0.9953	4
		50–1000[2]	$y = 0.3471 - 13.742$[b]	0.9897	
GSNO	−0.58	10–190[1]	$y = 0.04x + 0.141$[a]	0.9959	20
		50–1000[2]	$y = 0.0305x - 0.973$[b]	0.9888	

[a] Concentration of thiols was 200 μM and scan rate 25 mV s^{-1}.
[b] Concentration of thiols [1] μM; [2] nM.
[c] Current measurement [1] nA, [2] pA.
[d] Limit of detection calculated as 3 S/N.

(TCEP) is produced by Molecular Probes (Evgen, Oregon, USA). Sodium tetraborate and other used chemicals were purchased from Sigma–Aldrich. Stock standard solutions of GSH, GSSG and GSNO (100 mg ml^{-1}) were prepared by ACS water (Sigma–Aldrich, USA) and stored in the dark at the temperature of −20 °C. Working standard solutions were prepared daily by dilution of the stock solutions. The pH value was measured using WTW inoLab Level 3 with terminal Level 3 (Weilheim, Germany), controlled by the personal computer program (MultiLab Pilot; Weilheim, Germany). The pH-electrode (SenTix-H, pH 0–14/3 M KCl) was regularly calibrated by set of WTW buffers (Weilheim, Germany).

2.2. Electrochemical measurements

Electrochemical measurements were performed with the AUTOLAB Analyser (EcoChemie, Netherlands) connected to VA-Stand 663 (Metrohm, Switzerland), using a standard cell with three electrodes. The working electrode was a hanging mercury drop electrode (HMDE) with the drop area of 0.4 mm^2. The reference electrode was the Ag/AgCl/3 M KCl electrode and the auxiliary electrode was the graphite electrode. The analysed samples were deoxygenated prior to measurements by purging with argon (99.999%), saturated with water for 140 s. All experiments were carried out at room temperature. For smoothing and baseline correction [51], the software GPES 4.4 supplied by EcoChemie was employed.

2.2.1. Cyclic voltammetry measurements of GSH, GSSG and GSNO

The GSH, GSSG and GSNO were measured using normal cyclic voltammetry on HMDE. The supporting electrolyte (0.05 M sodium tetraborate, pH 9.2) from Sigma–Aldrich in ACS purity was purchased. CV parameters were as follows: the initial potential of −0.2 V, the end potential −0.8 V, scan rate 25 mV s^{-1} and step potential 5 mV. In the case of GSH and GSNO determination we used reducing agent TCEP (1 mM). For other experimental details see [50].

If we studied GSNO decomposition catalyzed by copper(II), we added solution of copper(II) and reducing agent TCEP (1 mM) to electrochemical cell, than, the solution was stirred in the electrochemical cell for duration of 140 s and subsequently the CV voltammogram was recorded as described above. If we studied the influence of iron(II) on the decomposition of GSNO, we used the same approach as in case of copper(II) excluding addition of TCEP.

2.3. Apolo 4000 system

NO selective carbon fibre electrode (ISO NOPF, 200 μm, WPI Europe) was connected to the Apolo 4000 instrument (WPI Europe) and inserted into distilled water for 30 min prior to the experiment. Calibration based on the copper(II) catalysed decomposition of *S*-nitroso-*N*-acetyl-*D*,*L*-penicillamine [52] (SNAP) was carried out according to instrument manual. Briefly: several aliquots of SNAP were sequentially added into continuously mixed solution of copper sulphate (100 mM, 10 ml, and 25 °C). Changes of the current were plotted against SNAP concentrations corrected by factor 0.6 which indicates effectiveness of copper(II) catalysed decomposition of SNAP.

In the case of studying of GSNO decomposition, borate buffer (50 mM, l.5 ml) containing 200 μM GSNO was placed into 2 ml plastic test tube wrapped into the aluminium foil to prevent light mediated decomposition of GSNO. Experiments were carried out at the temperature of 25 °C. The mixing of an analysed mixture was performed by a stirring bar (1000 rpm) and magnetic stirrer (MM 2A, Czech Republic). After baseline stabilization (from 5 to 10 min), stock solution of copper sulphate or ferrous sulphate were added to obtain desired final concentrations of copper(II) or iron(II). Current was recorded for 5 min after each addition. Experimental data were recalculated to the rate of NO releasing.

2.4. Statistical analysis

STATGRAPHICS® (Statistical Graphics Corp®, USA) was used for statistical analyses. Results are expressed as mean ± S.D. unless noted otherwise. Value of $p < 0.05$ was considered significant. Number of measurement is three unless noted otherwise.

3. Results and discussion

3.1. Cyclic voltammograms of GSH, GSSG and GSNO

Since discovery of glutathione, more than 80 years ago [1,53], this molecule has been still the object of study both for chemist and for biologist. Thanks to its SH group, GSH has been studied by polarographic methods [54–57]. Nowadays, it is possible to observe the marked increase of interest in an electrochemical study of thiol compounds including glutathione [39,58,59], first of all, due to the suggestion and development of new sensors, biosensors and biochips [36,60–67]. One of the interesting ability of glutathione is to bind number of biologically active molecules including heavy metals via SH groups [68–70]. It was published that nitric oxide could be bound on glutathione via sulfhydryl moiety forming *S*-nitrosoglutathione (GSNO; for chemical structure see Fig. 1A) [70]. In addition, nitric oxide could be released from GSNO via activity of number of different factors such as UV light, metal ions. Recently, we have observed the electrochemical behaviour of glutathione (GSH and GSSG) on the hanging mercury drop electrode in the presence of borate buffer and have studied the influence of tris(2-carboxyethyl)phosphine (TCEP) as a reducing agent by cyclic voltammetry [50]. This method does not require any derivatization reagent and even oxidized glutathione may be detected simultaneously [50,64]. Here we applied this technique for the analysis of GSH, GSSG and GSNO. The obtained voltammograms are shown in Fig. 1B. In the case of GSH, there are evident both reductive (at potential about −0.48 V) and oxidative (about −0.40 V) signals. The thirst signal at −0.48 V corresponds to reduction of GSHg

complex, which is formed according to reaction 1 [71,72]:

$$GSH + Hg \rightleftarrows GSHg + H^+ + e^- \quad (1)$$

Following the mechanism of GSSG reduction in cathodic part is suggested [54]:

$$GSSG + H^+ + e^- \rightleftarrows GSH + GS\cdot \quad (2)$$

$$GS^\bullet + H^+ + e^- \rightarrow GSH \quad (3)$$

In anodic part, there are two signals corresponding to: (1) formation of mercurous glutathionate, GSHg(I) – reaction 4; (2) oxidation of GSHg(I) to the mercuric glutathionate, GSHg(II) – reaction 5.

$$GSH + Hg - H^+ - e^- \rightarrow GSHg(I) \quad (4)$$

$$GSHg(I) - e^- \rightarrow GSHg(II) \quad (5)$$

GSNO embodies the similar shape of anodic part as GSSG, which indicate the possibility of formation GSH during cathodic part, where GSNO gave signal at potential of −0.58 V ($n = 10$; for anodic signals potentials of GSH, GSSG and GSNO see Table 1). As it was mentioned above, GSNO is very reactive and easily release NO from its structure. This reaction probably also takes place on the surface of the working hanging mercury drop electrode. During this process, formation of GSSG and its reduction in the anodic part could be expected. It clearly follows from the figure that height of GSH signal (P_{GSH}) is approximately three times higher than GSNO signal (P_{GSNO}). This phenomenon is probably caused by different kinetic properties of the particular electrode reactions.

3.2. Influence of scan rate on GSH, GSSG and GSNO signals

In addition, we studied the influence of scan rate on cathodic signals of P_{GSH}, P_{GSSG} and P_{GSNO}. To be specific, the height of the observed signals increased logarithmically with increasing scan rate (Fig. 1C). Moreover, the potential of the mentioned signals have not changed much according to increasing scan rate, except P_{GSH} signal shifted to more negative potentials. Other details will be published elsewhere.

3.3. Influence of different thiol concentrations on GSH, GSSG and GSNO signals

Moreover, the influence of different thiol concentrations on P_{GSH}, P_{GSSG} and P_{GSNO} signals at scan rate of 25 mV s^{-1} was studied. The concentration dependences were obtained by consecutive dilution of the thiols solutions at concentration of 200 μM. The calibration equations were linear, R.S.D. about 5%. (Table 1) The detection limits of GSH, GSSG and GSNO expressed as 3 S/N were 9, 4 and 20 nM, respectively; see Table 1. In spite of that the cyclic voltammetry do not allow to obtain a high sensitivity, it was possible to determine GSH, GSSG and GSNO at nanomolar concentration using the suggested approach. This phenomenon probably relates with formation a metal–thiol complex, which adsorb strongly at Hg surface [35,73,74] and, therefore, with pre-concentration of the thiol on the surface of HMDE due to relatively slow scan rate. It is absolutely clear that it will be necessary to use other electrochemical techniques and modifications to obtain higher sensitivity [35,74], this will be published elsewhere.

3.4. Detection of nitric oxide by NO selective carbon fibre electrode

After that we have observed the basic electrochemical behaviour of GSNO and have compared it with other thiols, we wanted to study the decomposition of GSNO through different electrochemical techniques. That is why, primarily, it was necessary to suggest not only the technique for the determination of GNSO but also method for quantification of nitric oxide, which is releasing in company with glutathione during decomposition of *S*-nitrosoglutathione. Recently, the simple apparatus consisting from carbon fibre electrode [75–77] and ion selective membrane [78] was developed for a determination of NO. The ion selective membrane enables nitric oxide to permeate it only [79,80]. The instruments is calibrated by *S*-nitroso-*N*-acetyl–*D*,*L*-penicillamine (SNAP) using copper as a catalyst [52]. The released NO from SNAP is amperometrically detected

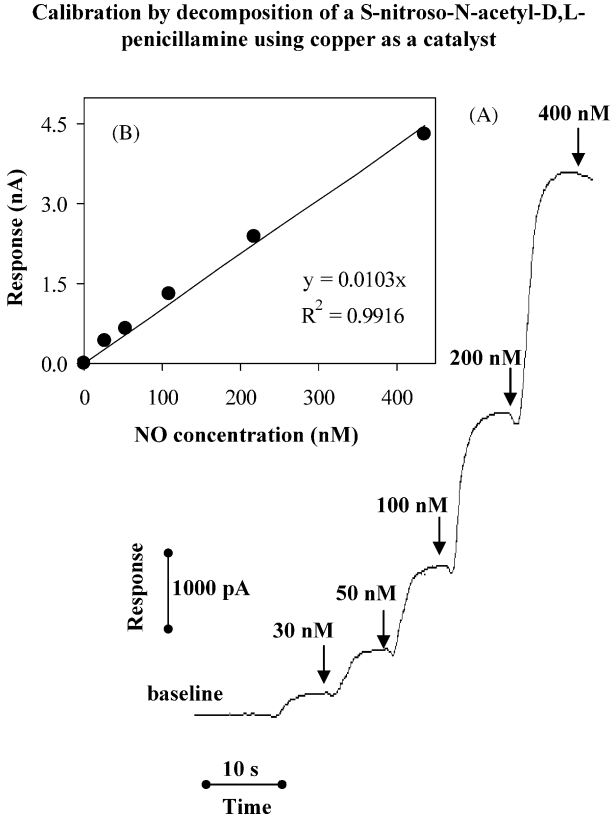

Fig. 2. Analysis of nitric oxide by NO selective carbon fibre electrode. (A) The instrument is calibrated by a donor of nitric oxide – *S*-nitroso-*N*-acetyl-*D*,*L*-penicillamine (SNAP) using copper(II) ($CuSO_4$) as a catalyst. The released NO from SNAP is amperometrically detected consequently. (B) Calibration dependence of nitric oxide. Amperometric parameters were as follows: detection potential of 850 mV, background electrolyte of 0.05 M borax ($Na_2B_4O_7$) at pH 9.2, stirring of 1000 rpm. For other details see Experimental section.

consequently at potential of 850 mV. The changes of current according to different concentration of releasing NO after addition of SNAP are shown in Fig. 2A. In addition the obtained calibration dependence was linear ($R^2 = 0.9916$; $n = 3$; R.S.D. = 2%; Fig. 2B) and detection limit of NO (3 S/N) was 2 nM. Now, we were able to study the changes of nitric oxide concentration by the above-mentioned apparatus in the following experiments, which is concerning study of GSNO decomposition.

3.5. Influence of copper on decomposition of GSNO

As we described above, the metal ions can release the nitric oxide from SNAP (Fig. 2); the scheme of releasing is shown in Eq. (6) [52,59]:

$$2\mathrm{RSNO} \rightarrow \mathrm{RSSR} + 2\mathrm{NO} \quad (6)$$

We assumed that the same effect of metal ions will be reached also at GSNO, which could serve as a NO donor. We were interested in the issue if it will be possible to observe the changes in P_{GSNO} according to additions of copper(II). Typical voltammogram of GSNO without copper(II) is shown in Fig. 3A (dotted line). We added consequently the different concentrations of copper(II) to GSNO. The signal of P_{GSNO} decreased with increasing concentration of copper(II), see Fig. 3B. To be specific, the P_{GSNO} signal decreased down to 50% in comparison with non-treated one at 20 nM copper(II) and, moreover, we observed other peak at potential −0.68 V, which corresponds probably to GSSG reduction (Fig. 3A, continuous line) [52]. Potential of P_{GSNO} shifted to more negative potential with increasing concentration of copper(II) (about 1 mV per change of copper(II) concentration by 1 nM, see inset in Fig. 3B). If we added 200 nM copper(II) to 200 μM GSNO, we observed only the peak of the oxidized glutathione (Fig. 3A, dashed line). We reduced disulfide bound by reducing agent of tris(2-carboxyethyl)phosphine (TCEP) according to [50] in order to ensure that the observed voltammogram (Fig. 3A, dashed line) really correspond to oxidized gluathione. If we added TCEP

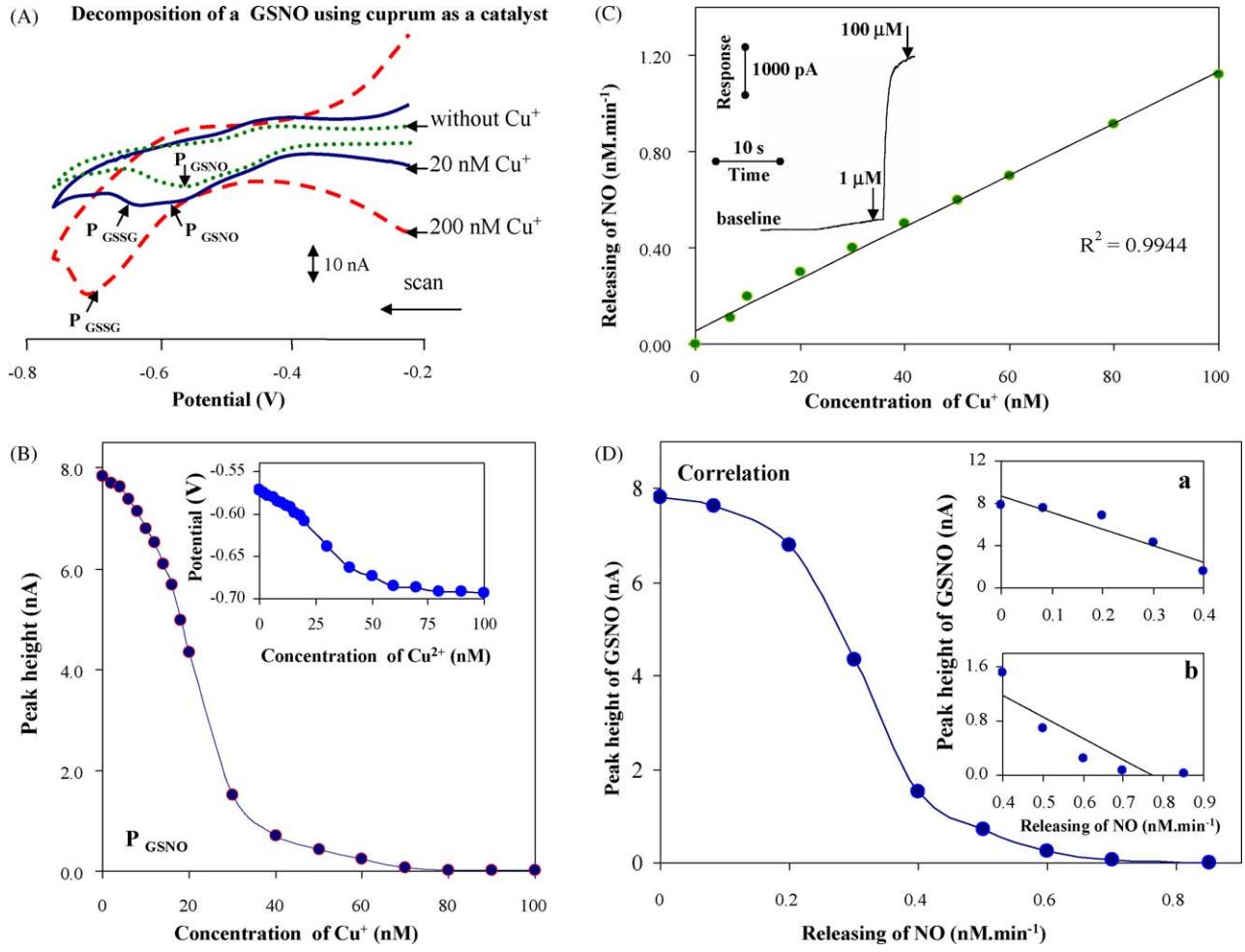

Fig. 3. Influence of copper(II) ($CuSO_4$) on decomposition of GSNO. (A) Typical cyclic voltammograms of the GSNO (200 μM) – GSNO without copper(II) (dotted line), GSNO with 20 nM copper(II) (continuous line) and GSNO with 200 nM copper(II) (broken line). The arrows are showing cathodic peaks of GSSG (P_{GSSG}) and GSNO (P_{GSNO}). (B) Changes of current response and potential of P_{GSNO} measured by cyclic voltammetry according to different concentration of copper(II) (0–100 nM). (C) Dependence of rate of nitric oxide releasing measured by NO selective carbon fibre electrode on different concentration of copper(II) (0–100 nM); in inset: changes of current response of nitric oxide at 1 μM and 100 μM of copper(II). (D) Correlation between releasing of nitric oxide and height of P_{GSNO} at concentration of copper(II) (0–100 nM); (Da) at concentration of copper(II) (0–30 nM); (Db) at concentration of copper(II) (30–100 nM). For other details see Figs. 1 and 2.

(1 mM), we obtained typical voltammetric curve corresponds to reduced glutathione (not shown). It clearly follows from the obtained results that two molecules of GSNO fall into two radicals of nitric oxide (•NO) and one molecule of GSSG according to assumed mechanism published in [52,81–84]. Particularly, It is well known that the decomposition of SNAP and GSNO occurs by a Cu^{+}catalyzed reaction pathway [52,82–84]. The mechanism now accepted is based on the reduction of Cu^{2+} by the thiolate anion RS^{-}, which is always present at low concentrations in solutions of RSNO. The sequence of reactions was published by Noble and Williams [85].

We used the method for NO determination we described in Section 3.4 to confirm the releasing of the nitric oxide from the GSNO molecule. Therefore, we added different concentrations of copper(II) to GSNO (200 μM) and observed the current changes. The addition of nanomolar concentrations of copper(II) (6–100 nM) caused low current responses, which corresponds to releasing of nitric oxide. The obtained dependence was linear ($y = 0.0108x + 0.0567$; $R^2 = 0.9944$, Fig. 3C). The huge releasing of nitric oxide from GSNO molecule was observed after the addition of copper(II) at micromolar concentrations (inset in Fig. 3C).

In addition, we wanted to correlate the decrease in GSNO signal measured by cyclic voltammetry with releasing of nitric oxide determined by amperometry at different concentrations of copper(II) (0–80 nM), see Fig. 3D. To be specific, the height of P_{GSNO} decreased slowly in the concentration range of copper(II) from 0 to 10 nM. Furthermore, we observed the marked decrease of P_{GSNO} height up to 30 nM of copper(II) by the CV method, which relates with increasing rate of decomposition of GSNO. The correlation between height of P_{GSNO} and releasing of NO at concentration of copper(II) from 0 to 80 nM is shown in Fig. 3Da. This correlation dependence was plotted by straight line ($y = -15.715x + 8.7165$; $R^2 = 0.8858$). In addition the height of P_{GSNO} did not change much at the concentration of copper(II) higher than 30 nM. If we plotted the correlation

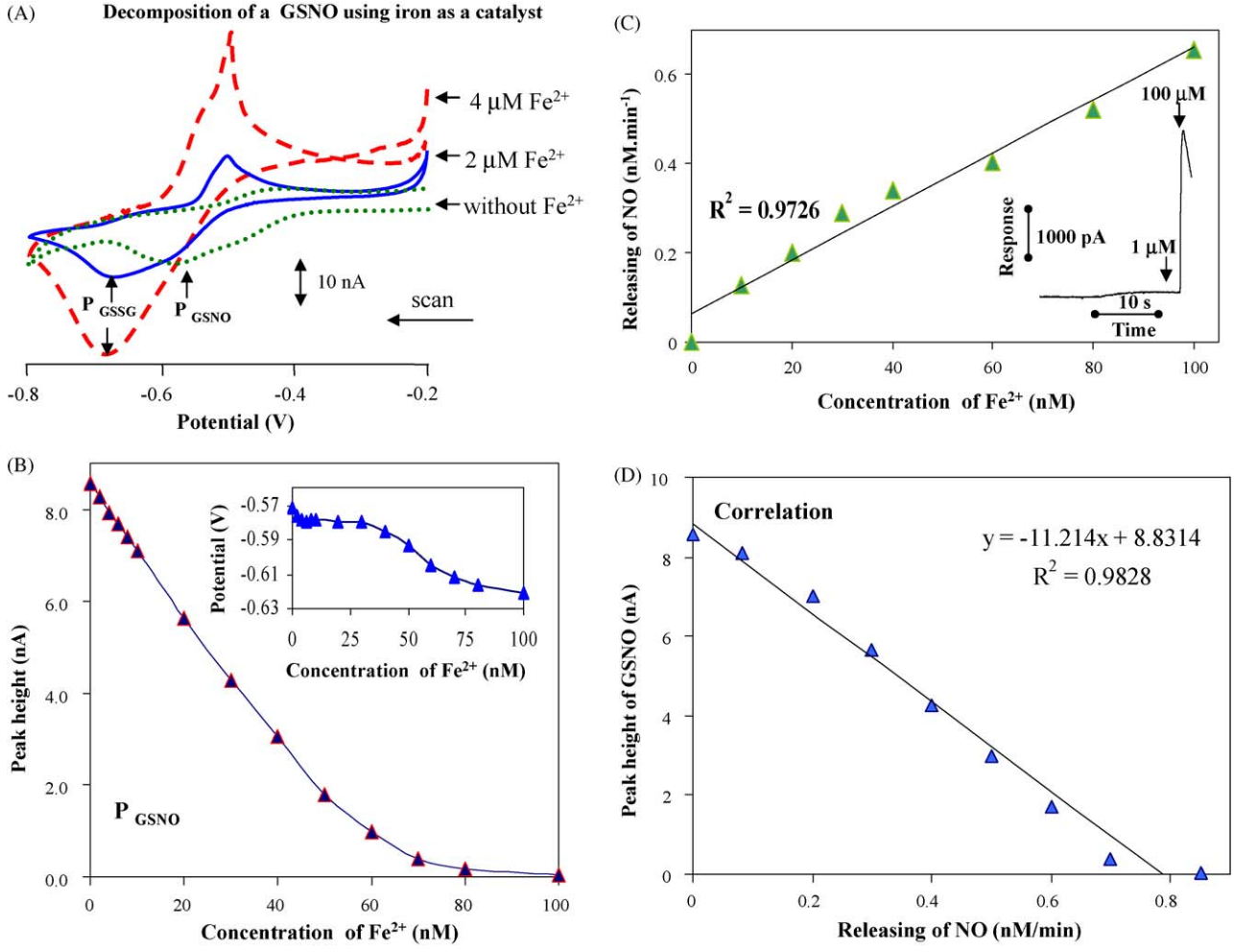

Fig. 4. Influence of iron(II) ($FeSO_4$) on decomposition of GSNO. (A) Typical cyclic voltammograms of the GSNO (200 μM) – GSNO without iron(II) (dotted line), GSNO with 2 μM iron(II) (continuous line) and GSNO with 4 μM iron(II) (broken line). The arrows are showing cathodic peaks of GSSG (P_{GSSG}) and GSNO (P_{GSNO}). (B) Changes of current response and potential of P_{GSNO} measured by cyclic voltammetry according to different concentration of iron(II) (0–100 nM). (C) Dependence of rate of nitric oxide releasing measured by NO selective carbon fibre electrode on different concentration of iron(II) (0–100 nM); in inset: changes of current response of nitric oxide at 1 and 100 μM of iron(II). (D) Correlation between releasing of nitric oxide and height of P_{GSNO} at concentration of iron(II) (0–100 nM). For other details see Figs. 1 and 2.

between height of P_{GSNO} and releasing of NO at concentration of copper(II) from 30 to 80 nM, we obtained this equation $-y = -4.423x + 3.0886$; $R^2 = 0.9208$ (Fig. 3Db).

3.6. Influence of iron on decomposition of GSNO

We assumed that the iron(II) would have the similar effect on the decomposition of the GSNO as the copper(II). Typical cyclic voltammograms of GSNO with or without iron(II) are shown in Fig. 4A. The height of P_{GSNO} linearly decreased according to increasing concentration of iron(II) up to 60 nM and then did not change much (Fig. 4B). The potential of the mentioned peak shifted to more negative potential with increasing iron(II) concentration (inset in Fig. 4B). On the base of the obtained results we assumed that decomposition of GSNO catalysed by iron(II) proceeds following Eq. (7):

$$2\text{GSNO} + \text{Fe}^{2+} \rightarrow \text{GSSG} + \text{Fe}^{2+} + 2^{\bullet}\text{NO} \quad (7)$$

As we mentioned above, if we wanted to confirm the releasing of the nitric oxide from the GSNO molecule catalysed by iron(II), we used NO selective carbon fibre electrode. The dependence of releasing of nitric oxide on concentration of iron(II) was linear ($R^2 = 0.9726$; Fig. 4C). In addition, the releasing of nitric oxide from the GSNO catalysed by iron(II) was slower (approximately two-times) in comparison with the copper(II). The paper from Williams group [86] shows that Fe^{2+} catalyzes the release of NO from RSNO to a lesser extent than Cu^{+} which is in accordance with the results presented in our manuscript. If we wanted to obtain the same rate of releasing of nitric oxide from GSNO as in the case of copper(II), we had to use the concentration of iron(II) about 40% higher than that of copper(II). Moreover, the correlation between decrease in height of P_{GSNO} and releasing of NO at different concentrations of iron(II) is shown in Fig. 4D. It clearly follows from the figure that the dependence is approximately linear ($y = -11.214x + 8.8314$; $R^2 = 0.9828$), which is probably relates with lower efficiency of iron(II) as catalyst of the GSNO decomposition in comparison with copper(II).

4. Conclusion

New tools for determination of nitroso-compounds, which are donors of nitric oxide in plant and animal tissues, have been decribed. Particularly, we suggest two different electrochemical techniques that are possible to use both for studying of amounts of nitric oxide and *S*-nitrosoglutathione and for observing of decomposition of a nitroso-compounds.

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