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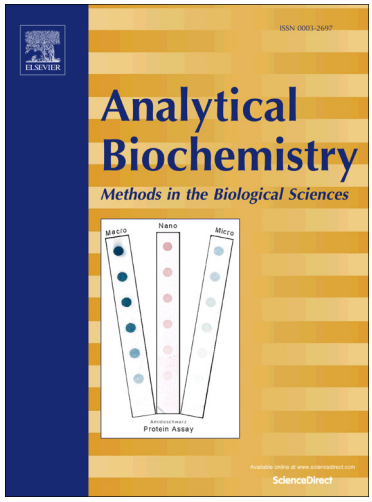

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Electrochemical assay for the determination of nitric oxide metabolites using copper(II)chlorophyllin modified screen printed electrodes

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Abstract

This work presents a novel electrochemical assay for the collective measurement of nitric oxide (NO) and its metabolites nitrite (NO_2^-) and nitrate (NO_3^-) in volume miniaturized sample at low cost using copper(II) chlorophyllin (CuCP) modified sensor electrode. Zinc oxide (ZnO) incorporated screen printed carbon electrode (SPCE) was used as a host matrix for the immobilization of CuCP. The morphological changes of the ZnO, CuCP modified electrodes were investigated using scanning electron microscope. The electrochemical characterization of the CuCP-ZnO-SPCE exhibited the characteristic quasi-reversible redox peaks at the potential, +0.06 V *vs.* Ag/AgCl. This biosensor electrode showed a wide linear range of response over NO concentrations from 200 nM to 500 µM with a detection limit of 100 nM and sensitivity of 85.4 nA μM^{-1}. Further, NO_2^- measurement showed the linearity of 100 nM to 1 mM with a detection limit of 100 nM for NO_2^- and the sensitivity of 96.4 nA μM^{-1}. Then, the concentration of NO_3^- was measured after its enzymatic conversion into NO_2^-. Using this assay, the concentrations of NO, NO_2^- and NO_3^- present in human plasma samples before and after beetroot supplement were estimated using suitable membrane coated CuCP-ZnO-SPCE and validated with the standard Griess method.

Keywords

Nitric oxide; Nitrite; Nitrate; Copper(II) chlorophyllin; Electrochemical assay, Screen printed carbon electrode.

1. Introduction

Nitric oxide (NO) acts as a biological signaling molecule in many physiological and pathological processes [1]. It possesses multiple vasoprotective characteristics, including vasorelaxation, inhibition of endothelial cell apoptosis, platelet aggregation, leukocyte chemotaxis and SMC proliferation and migration [2, 3]. Endogenous NO metabolism produces the oxidative metabolites nitrite (NO_2^-) and nitrate (NO_3^-) and they are emerged as an endocrine reservoir capable of producing NO within hypoxic, ischemic or injured tissue [4]. The nitrate-nitrite-derived NO has been involved in conferring cytoprotection in a number of animal models of diseases, limit I/R-induced apoptosis and modulate cell signaling and tissue responses to hypoxia [5]. Therefore, the determination of NO and its metabolites NO_2^- and NO_3^- are becoming more significant in human physiology during hypoxia, nutrition and therapeutics.

Several analytical techniques such as Griess colorimetric assay [6, 7], fluorometry [8] and chemiluminescence [9] have been widely reported for the determination of NO, NO_2^- and NO_3^-. Separation based methods include GC-MS [10, 11] and HPLC [12, 13] with variety of detection system are also reported for the determination of NO_2^- and NO_3^-. The short half-life of NO (easily gets oxidized in the presence of oxygen) and its low concentration reduces the practicality of these methods. Usually they have tedious detection procedures and therefore are time-consuming. Recently, electrochemical biosensor technique is proved to be an appropriate alternative by providing practical advantages, such as operation simplicity, low expense of fabrication and suitability for real-time detection. In addition, it gives fast response, more sensitive (particularly with the use of modified electrodes) and selective determination.

In the literature, numerous electrochemical biosensors were reported for the independent determination of NO, NO_2^- and NO_3^- [14-19]. However, they are limited for the biological samples in terms of required large volume and also able to measure only single analyte in an experiment. The analytical applicability of the above reported biosensors for the biological samples were quite restricted since it requires minimum 1 mL of the sample for the measurement. Moreover, it is difficult for the researchers to collect serum/plasma in large volume from blood. Therefore, there is a real need for the measurements of these clinically important analytes collectively in a volume miniaturized sample. In order to minimize the sample volume, researchers are focusing on screen printed carbon electrode (SPCE) because of their unique properties such as small size, low detection limit, fast response time and high reproducibility [20-23]. Further, it allows the mass production of electrochemical biosensors at low cost in comparison to other usual electrodes [24-27].

Our earlier research focused on the enzymatic determination of NO, NO_2^- using copper, zinc superoxide dismutase (SOD1) and NO_3^- using nitrate reductase (NaR) modified Pt electrodes [28-30]. The enzymes have less stability, high cost, tedious procedure and time-consuming. Inorder to resolve these drawbacks, we have used here the nanocomposite of metalloporphyrins and zinc oxide (ZnO). Structure–activity relationships have guided the development of positively, negatively charged and neutral metalloporphyrins. A wide range of different metalloporphyrins including nickel [31–34], iron [35-38], cobalt [39,40] manganese [41-43] and some other metal complexes [44,45] have been found to serve as effective catalysts for the oxidation or reduction of NO and other analytes. Here, the copper (II) chlorophyllin (CuCP) contains the copper at the centre of the porphyrin with excellent electrocatalytic redox property as similar to that of SOD1. Furthermore, due to its high stability and low cost, it is a preferred

target for the fabrication of electrochemical sensors. Nanostructured metal oxides such as ZnO have recently been used for fabrication of transducer surface because of their unique ability to promote faster electron transfer between electrode and active site of desired enzyme mimetic. Furthermore, nontoxicity, high chemical stability and high electron transfer capability make ZnO a promising material for immobilization of desired biomolecules without electron mediator. Among the semiconducting metallic oxides, ZnO can easily be electrochemical deposited (ECD, also termed electrodeposition) at low temperature, to give nanostructures of ZnO matrix. This method based on the soft chemical approach has also triggered great interest in recent years due to its low cost, large-scale deposition, and unique ability to tune the orientation and morphology of electrodeposited films compared with other methods. Owing to its biocompatibility with high electro communication features, the nanoparticles of ZnO was immobilized with biomimetic CuCP possessing an interesting application in biosensor due to the synergic effect of ZnO and CuCP. Electrochemical and electrocatalytic properties of a CuCP-ZnO film coated electrode were not reported. In the present paper, for the first time, we have deposited CuCP on to ZnO film using electrochemical technique. The nano ZnO-CuCP modified electrode was used to determine NO at a relatively low detection limit and with improved sensitivity. The sensor exhibited excellent performance features, such as low detection limits, wide linear range, quick current response, enhanced sensitivity and good stability.

Further, the possible interferences present in the biological samples were eliminated by using nafion (selective for NO) and cellulose acetate membranes (selective for NO_2^- & NO_3^-). The concentrations of NO and NO_2^- were measured as present in the sample whereas NO_3^- was measured after its enzymatic conversion into NO_2^- using NaR. Using this highly sensitive and selective electrochemical sensor the concentrations of NO and NO_2^- & NO_3^- present in the human

plasma samples before and after the beetroot supplementation were estimated and validated with the standard Griess method.

2. Material and methods

2.1. Reagents

Sodium ascorbate, uric acid, sodium hydrogen phosphate, disodium hydrogen phosphate, citric acid, nafion, cellulose acetate, zinc nitrate, urea, NaOH, H_2SO_4, $NaNO_2$, $NaNO_3$, KCl and glutaraldehyde were obtained from Sigma Company (Milwaukee, WI, USA). Copper(II) chlorophyllin trisodium was obtained from Alfa Aesar. All the solutions were prepared with doubly distilled water.

2.2. Instrumentations

All the electrochemical experiments were performed using CHI 1200B electrochemical workstation (CH Instruments, USA) with a conventional three electrode system. A three electrode type of screen printed carbon electrodes (SPCE, CH Instruments, USA) were used for the biosensors fabrication. The working electrode, counter electrode and the reference electrode of the SPCE are carbon and Ag/AgCl electrode respectively. The surface of the working electrode is 0.071 cm^{-2}. The morphological scanning electron microscopic (SEM) images and energy dispersive X-ray analysis (EDX) spectra were obtained using a FEI Quanta FEG 200-High Resolution Scanning Electron Microscope (FEI Co., Netherlands).

2.3. Preparation of saturated NO solution

Saturated NO solution (2 mM) was prepared as per earlier report [46]. Briefly, NO gas was produced by dropping 1.5 M sulfuric acid solution in saturated solution of sodium nitrite ($NaNO_2$). Then, the produced NO gas was forced to bubble into a NaOH solution (30%) to remove the NO_2 (formed as a result of its reaction with O_2) and the other gases. This filtered

pure NO gas was bubbled in water for 10 min. Further, this saturated NO solution was used for the various concentration study by making serial dilutions. All the solutions used for the preparation of standard (including water for dilutions) and buffer solutions were deoxygenated with argon for 15 min.

2.4. Measurement of NO_2^- and NO_3^- levels in human plasma

The studies were granted full ethics approval by the Institutional Research Ethics Committee, and all subjects gave informed consent. All the healthy volunteers (non-smokers, avg. age 26 ± 2 years, n = 4) received 500 ml of beet root juice (Defence Food Research Laboratories, Mysore) for five consecutive days. Blood samples were collected (5 ml each) in citrate tubes before beet root juice supplementation and after 5 days of supplementation, centrifuged at 2200 g for 10 minutes at 4 ºC. The plasma was collected, alliquoted and one aliquot were passed through 10 KDa cut off filters and NO_3^- + NO_2^- (NOx) levels were measured after reducing NO_3^- to NO_2^- using a colorimetric kit (Cayman Chemicals, USA). The second aliquot was used for the determination of NO_3^- and NO_2^- using the developed biosensor.

2.5. Construction of CuCP-ZnO-SPCE

Prior to fabricate the biosensor electrode, the SPCE was pre-treated as per earlier report [47] to remove the organic ink constituents or contaminants and to increase the surface functionalities. Briefly, SPCE was dipped in 0.1 M PBS solution and cycling the potential from -0.6 to +1.6 V (*vs.* Ag/AgCl) for 40 cycles at a scan rate, 10 mVs^{-1}. After pre-treatment, ZnO was incorporated by placing the mixture of 0.1 M zinc nitrate and 0.5 M urea solution on the working electrode surface of the SPCE and cycling the potential from -1 to +1 V for 10 complete cycles. Then, CuCP modified ZnO-SPCE (scheme 1) was prepared by placing the mixture of 0.1 M CuCP, NaOH and KCl solution onto the ZnO incorporated SPCE and cycling the potential from -1 to +1

V for 10 cycles. During this process, CuCP was electrodeposited on the ZnO-SPCE. It was then gently washed with 0.1 M PBS and stored at 4 ºC when not in use.

Possible position for Scheme 1

The above fabricated biosensor electrode CuCP-ZnO-SPCE were highly reproducible, which could be confirmed from the cyclic voltammetric responses. All the experiments were carried out at 27±0.5 ºC.

2.6. Results and discussion

The available methods are able to measure the concentration of NO and its metabolites *viz.*NO_2^- and NO_3^- independently and also required large volume. Therefore, there is a real need for the development of alternative assay for the collective measurement of these three analytes in volume miniaturized sample at low cost with high sensitivity and selectivity. Hence, the aim of this work was to exploit the electrochemical assay kit for the rapid determination of NO and its metabolites in one drop of a sample using CuCP modified ZnO-SPCE. The electrochemical responses of the CuCP-ZnO-SPCE for the determination of NO, NO_2^- and NO_3^- were investigated. In order to test the performance of the developed electrochemical assay kit, sensitivity, selectivity, life time, reproducibility, effect of pH and scan rate were investigated.

SEM and electrochemical characterization of the modified biosensor electrodes

Possible position for Fig. 1

The morphological images of the bare SPCE (a), ZnO-SPCE (b) and CuCP-ZnO-SPCE (c) and its energy dispersive X-ray analysis (EDX) spectra obtained using SEM. Fig. 1a represents the typical highly porous morphology of bare SPCE. Fig.1b demonstrates the incorporation of ZnO on SPCE surface respectively. Fig. 1c clearly reveals the distribution of CuCP on the ZnO-SPCE and further its elemental analysis spectrum confirmed the existence of Cu and Zn on SPCE.

Fig. 2 shows the typical electrochemical responses obtained for the bare SPCE (curve a), ZnO-SPCE (curve b) and CuCP-ZnO-SPCE (curve c) using a scan rate of 50 mVs^{-1} in 0.1 M PBS (pH 7.0). There were no peaks obtained for the bare SPCE and ZnO-SPCE, however the CuCP-ZnO-SPCE exhibited the quasi-reversible redox peak at the potential, -0.4 V *vs.* Ag/AgCl. This observed quasi-reversible peak perhaps attributed to the Cu^{2+}/Cu^{+} redox changes at the active site of the CuCP.

Possible position for Fig. 2

2.6.1. Effect of pH and scan rate

The electrochemical behaviors of the CuCP-ZnO-SPCE were investigated in the pH range of 3.0-10.0. For the pH study, a mixture of disodium hydrogen phosphate and citric acid buffer was used. The current responses of both the biosensor electrodes were decreased from pH 7.0 - 3.0 and also from pH 7.0 - 10.0, perhaps due to the denaturation of immobilized CuCP. However, the maximum current response was observed at pH 7.0 (Fig. 3A). Further, the influence of the scan rate on the CV performance of the CuCP-ZnO-SPCE was investigated. It was found that the anodic peak currents negatively increased linearly with increasing the scan rate from 50 to 300 mVs^{-1} and as well as the characteristic of CV remains unchanged. These indicate the favorable orientation of CuCP on the ZnO-SPCE (Fig. 3B).

Possible position for Fig. 3A

Possible position for Fig. 3B

2.6.2. Electrochemical response to NO and NO_2^-

Fig. 4A exhibits the typical electrochemical responses obtained for the CuCP-SPCE and CuCP-ZnO-SPCE in the absence (curve a & curve b) and presence (curve c & curve d) of 100 µM NO using a scan rate of 50 mVs^{-1} in 0.1 M PBS (pH 7.0). Before the addition of NO, there were no changes observed in the current response. However, after the addition of NO, both the CuCP-

SPCE and CuCP-ZnO-SPCE exhibited the significant increases in current anodically at the potential, 0.8 V. Further, it is clearly seen that the CuCP-ZnO-SPCE (curve d) shows the higher current response than CuCP-SPCE (curve c). It is perhaps due to the n-type semiconducting ZnO enhanced the electron transfer between the active site of the porphyrin CuCP and the electrode surface during the oxidation of NO *via* a cyclic redox reaction of its Cu(I/II) active site moiety (scheme 1). Fig. 4B illustrates the electrochemical responses obtained for the CuCP-ZnO-SPCE in the presence of various NO concentrations at the same scan rate. The observed anodic peak currents *vs.* NO concentrations were plotted as shown in inset of Fig. 4B. The calibration curve obtained exhibits a linear range of response over the concentration of NO from 100 nM to 500 µM but for clarity here we have shown from 50 µM to 500 µM (r^2 = 0.9968, n=3) with a detection limit of 100 nM and sensitivity of 85.4 nAµM^{-1}.

Possible position for Fig. 4A and 4B

Fig. 5A represents the typical CV responses obtained for the CuCP-SPCE and CuCP-ZnO-SPCE in the absence (curve a & curve b) and presence (curve c & curve d) of 100 µM NO_2^- using a scan rate of 50 mVs^{-1} in 0.1 M PBS (pH 7.0). Before the addition of NO_2^-, there are no changes observed in the current response. However, after the addition of NO_2^-, both the CuCP-SPCE and CuCP-ZnO-SPCE exhibited the significant increases in current anodically at the potential, 0.83 V. The electrochemical responses of the CuCP-ZnO-SPCE at the various concentrations of NO_2^- are shown in Fig. 5B and its linear calibration was plotted as shown in inset of Fig. 5B. The calibration curve thus obtained exhibits a linear range of response over the concentration of NO_2^- from 100 nM to 1 mM but we have shown here for clarity from 100 µM to 1 mM (r^2 = 0.9984, n=3) with a detection limit of 100 nM and sensitivity of 96.4 nA μM^{-1}.

Possible position for Fig. 5A and 5B

2.6.3. Elimination of interferences using nafion and cellulose acetate membranes

NO_2^- is a main source of interference in the determination of NO because it is one of the oxidation products of NO and oxidized at nearly the same potential. Besides, the common co-existing biological substrates such as ascorbic acid, uric acid may also interfere with the NO measurement. In order to eliminate all these possible interferences, nafion membrane was used. Nafion membrane coated CuCP-ZnO-SPCE was made by dropping 10 µL of nafion solution (nafion in ethanol) onto CuCP modified ZnO-SPCE and dried at room temperature for 10 min. Fig. 6 illustrates the electrochemical responses obtained for the CuCP-ZnO-SPCE before and after nafion coating in (i) 0.1 M PBS containing 100 µM NO (curve b & c) and (ii) 0.1 M PBS containing 100 µM NO_2^- (curve a & d). Prior to nafion coating, CuCP-ZnO-SPCE exhibited the electrochemical oxidation of both NO (curve c) and NO_2^- (curve d) at nearly the same potential. However, after nafion coating, the CV responses of CuCP-ZnO-SPCE (curve b) for NO was decreased to 60.6% (i.e. the current response was decreased from -19.5 µA to -11.8 µA) and at the same time the response for NO_2^- was almost not observed (curve a).

Possible position for Fig. 6

Similarly, in order to measure NO_2^- selectively, cellulose acetate membrane was coated on the CuCP-ZnO-SPCE by dropping the 10 µL of cellulose acetate solution (cellulose acetate in acetone) and dried at room temperature. After cellulose membrane coating, the CV response for NO_2^- was decreased to 70.3% (i.e. the current response was decreased from -23.2 µA to -16.3 µA) and further the responses for the interferences ascorbic acid, uric acid were almost not observed (data not shown). Both the nafion and cellulose acetate membranes were not only exclude the interferences and also prevent the electrodes from fouling due to the non-specific adsorption of proteins and other materials typically present in biological samples.

2.6.4. Stability and reproducibility

In order to check the stability and reproducibility of the CuCP-ZnO-SPCE, the anodic current responses for the NO, NO_2^- were recorded three times daily. The observed current responses were found to be constant for two months for the CuCP-ZnO-SPCE. Therefore CuCP modified SPCE shows the longer stability in table 1.

2.6.5. Electrochemical measurement of NO level in human plasma

The utility of the developed electrochemical assay kit for the measurement of NO in human plasma was tested. Nafion membrane coated CuCP-ZnO-SPCE was used for the determination of NO. One aliquot of plasma sample was dropped on the working electrode surface of the SPCE and the corresponding current response was observed at 0.8 V. The obtained results were shown in table 2. The measured values of NO levels present in the human plasma samples of normal human subject was 250 ± 13 nM. The measured values are in good agreement with the previous reported data [48].

2.6.6. Electrochemical measurement of total NO_2^- and NO_3^- level in human plasma

The measurement of total NO_2^- and NO_3^- in blood is an index of endothelial nitric oxide synthase activity [49]. Moreover, the high altitude subjects have suffered by the reduction of NO_2^- level in their blood leading to several diseases at hypoxia condition [50]. Recent study reported that the administration of NO_3^- rich beetroot juice to human and several animal models promotes NO-like bioactivity and regulates biological activities like reduction of blood pressure, vasodilation, cytoprotection, cardioprotection, protection from ischemia-reperfusion injury [51-53]. Therefore, in this work, we have attempted to measure the total NO_2^- and NO_3^- levels in human plasma of four subjects before and after beetroot supplementations. The CuCP modified SPCE were coated with cellulose acetate membrane and employed to estimate the total NOx (NO_2^- + NO_3^-) concentration. The results were obtained by dropping one drop of the plasma sample on the

working electrode surface and shown in table 3. The concentration of NO_2^- was also measured in the sample before the enzymatic reduction of NO_3^- into NO_2^-. Further, the concentration of NO_3^- was estimated by subsequent subtraction of $[NO_2^-]$ from the total $[NO_2^- + NO_3^-]$.

$$[NO_3^-] = [NO_2^- + NO_3^-] - [NO_2^-]$$

It could be concluded from the table 2 that the administration of exogeneous NO_3^- increased the concentration of total NO_2^- and NO_3^- value in blood thereby enhanced the $NO\text{-}NO_2^-\text{-}NO_3^-$ pathway.

2.7. Conclusions

We have demonstrated here the highly sensitive and selective electrochemical assay kit for the collective measurement of NO, NO_2^- and NO_3^- in volume miniaturized sample using CuCP modified ZnO-SPCE as a novel electrochemical biosensor. The electrocatalytic activity of CuCP towards the oxidation of NO and NO_2^- were investigated. However, CuCP-ZnO-SPCE was more stable and reproducible. Further, high selectivity was achieved by using nafion membrane for NO and cellulose acetate membrane for NO_2^- and NO_3^-. Using this electrochemical assay kit, the concentrations of NO and its metabolites NO_2^- and NO_3^- present in the human plasma samples were estimated and correlated with the standard Griess method.

Abbreviations

CV	Cyclic Voltammetry
CuCP	Copper (II) chlorophyllin
EDX	Energy dispersive X-ray spectroscopy
I/R-induced	Ischemia/reperfusion-induced
PBS	Phosphate buffer solution
SEM	Scanning electron microscopy
SPCE	Screen printed carbon electrodes
NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
$NaNO_2$	Sodium nitrite
NaR	Nitrate reductase
ZnO	Zinc oxide

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Figure caption

Scheme 1: Schematic representation of the fabrication of CuCP-ZnO-SPCE and the illustration of the biochemical reaction occurring at the biosensors surface during the determination of NO, NO_2^-.

Fig. 1: SEM images of (a) bare SPCE, (b) ZnO-SPCE, (c) CuCP-ZnO-SPCE and its EDX spectrum of the electrodes.

Fig. 2: Typical CV responses of (a) bare SPCE, (b) ZnO-SPCE, and (c) CuCP-ZnO-SPCE electrodes in 0.1 M PBS at pH 7.0 at scan rate: 50 mVs^{-1} *vs* Ag/AgCl.

Fig. 3A: Effect of pH on peak current of CuCP-ZnO-SPCE electrodes in 0.1 M PBS at scan rate: 50 mVs^{-1} *vs* Ag/AgCl. Each point represents the average of three measurements.

Fig. 3B: Effect of increasing scan rate from 50 to 300 mVs^{-1} on CuCP-ZnO-SPCE in 0.1 M PBS solution.

Fig. 4A: Electrochemical responses obtained for the CuCP-SPCE and CuCP-ZnO-SPCE in the absence (curve a and b) and presence (curve c and d) of 100 µM NO solution at scan rate: 50 mV s^{-1} *vs.* Ag/AgCl.

Fig. 4B: Electrochemical responses obtained for the CuCP-ZnO-SPCE in the presence of (a) control, (b) 50 µM, (c) 100 µM, (d) 200 µM, (e) 300 µM, (f) 400 µM and (g) 500 µM of NO solution at scan rate: 50 mVs^{-1} *vs.* Ag/AgCl. *Linear calibration curve (inset of Fig. 4B)* $y = -0.0983x - 10.019$, $r^2 = 0.9968$.

Fig. 5A: Electrochemical responses obtained for the CuCP-SPCE and CuCP-ZnO-SPCE in the absence (curve a and b) and presence (curve c and d) of 100 µM NO_2^- solution at scan rate: 50 mVs^{-1} *vs.* Ag/AgCl.

Fig. 5B: Electrochemical responses obtained for the CuCP-ZnO-SPCE in the presence of (a) control, (b) 100 µM, (c) 300 µM, (d) 400 µM, (e) 500 µM, (f) 600 µM and (g) 1000 µM of NO_2^- solution at scan rate: 50 mVs^{-1} *vs.* Ag/AgCl. *Linear calibration curve (inset of Fig. 5B)* $y = -0.0962x-10.269$, $r^2 = 0.9984$.

Fig. 6: Electrochemical responses obtained for the CuCP-ZnO-SPCE before and after nafion coating in (i) 0.1 M PBS containing 100 µM NO (curve b & c) and (ii) 0.1 M PBS containing 100 µM NO_2^- (curve a & d).

Table 1: Electroanalytical properties of CuCP-ZnO-SPCE

Table 2: Electrochemical measurement of NO in human plasma

Table 3: Electrochemical measurement of total NO_2^- + NO_3^- levels in human plasma before and after beetroot supplementation

Electrochemical assay for determination of nitric oxide metabolites using copper(II) chlorophyllin modified screen printed electrodes.

Murugesan Balamurugan[1], Thangamuthu Madasamy[1], Manickam Pandiaraj[1], Paulraj Santharaman [1],Kalpana Bhargava[2], Niroj Kumar Sethy[2], Chandran Karunakaran[1*]

Figure 1

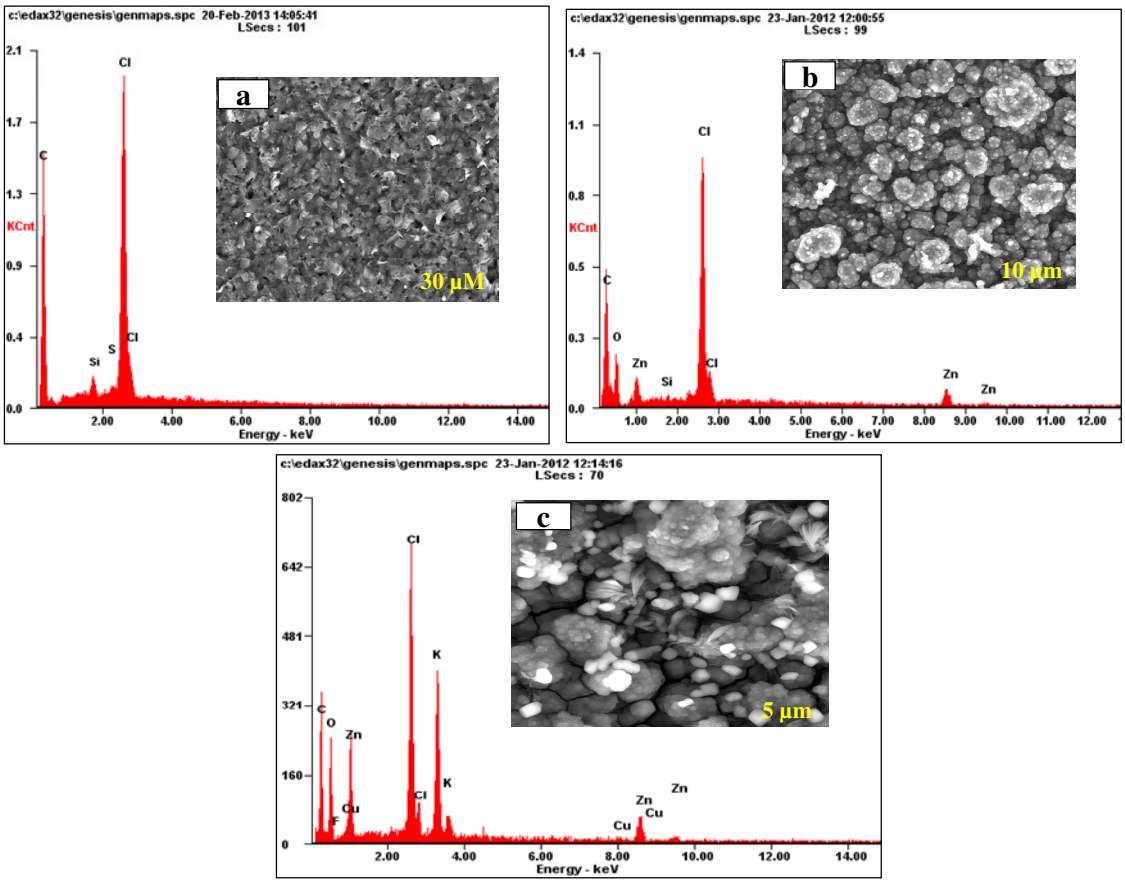

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Figure 2

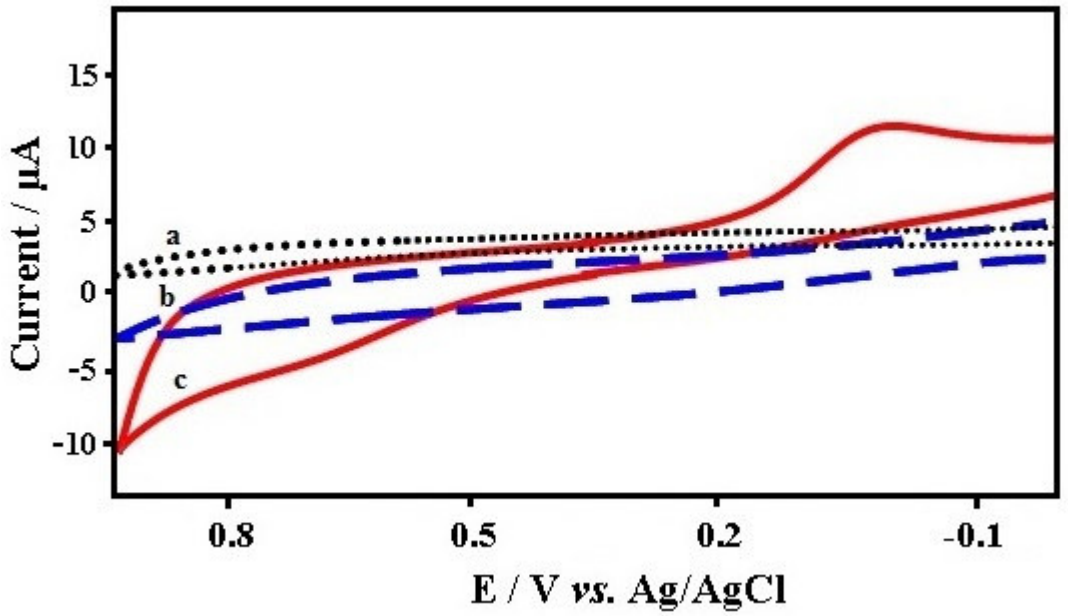

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Figure 3A

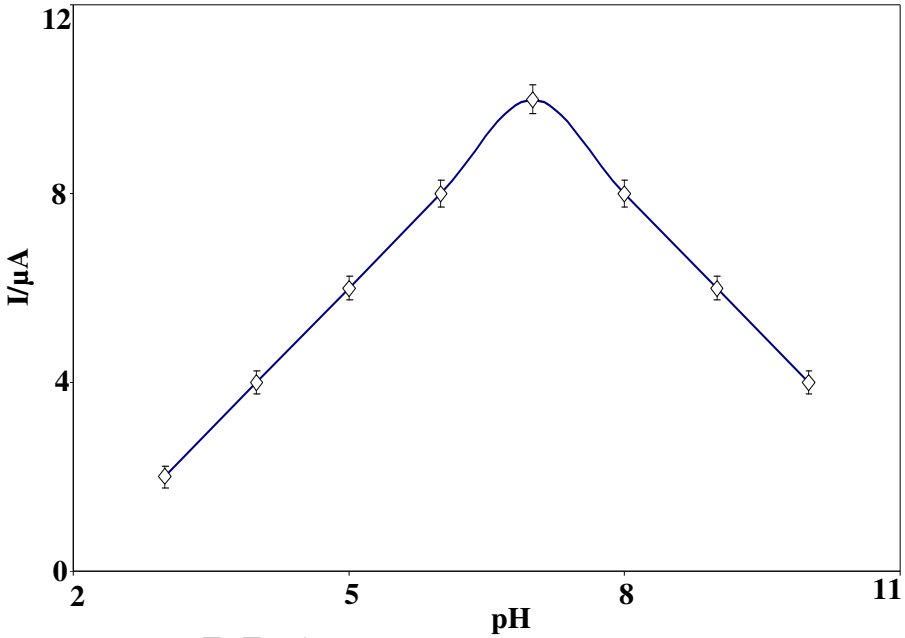

Fig. 3A: Effect of pH on peak current of CuCP-ZnO-SPCE electrodes in 0.1 M PBS at scan rate: 50 mVs^{-1} *vs* Ag/AgCl. Each point represents the average of three measurements.

Figure 3B

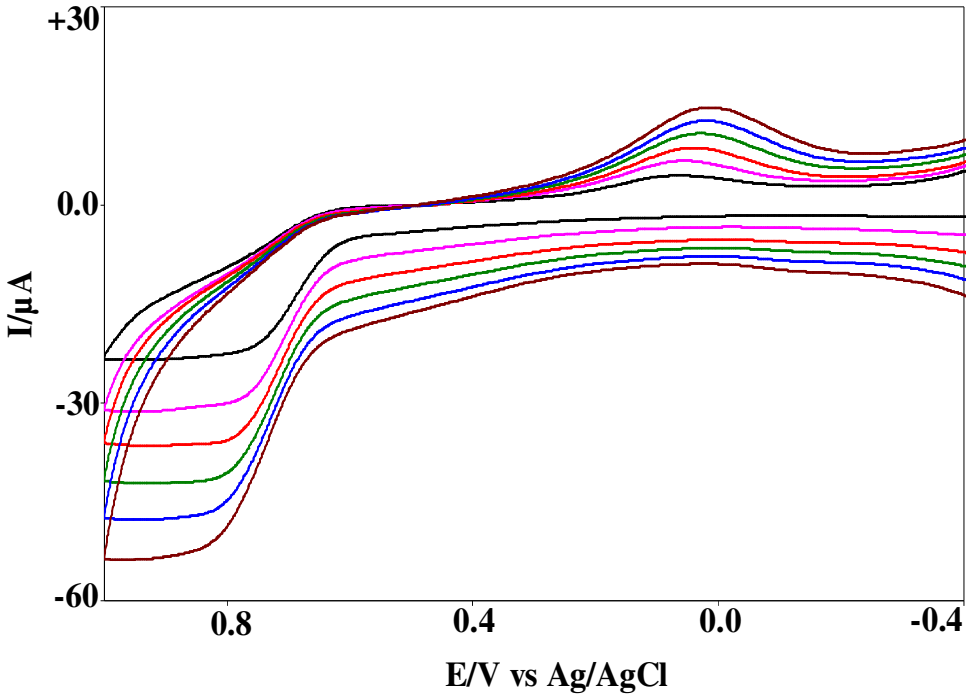

Fig. 3B: Effect of increasing scan rate from 50 to 300 mVs^{-1} on CuCP-ZnO-SPCE in 0.1 M PBS solution.

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Figure 4A

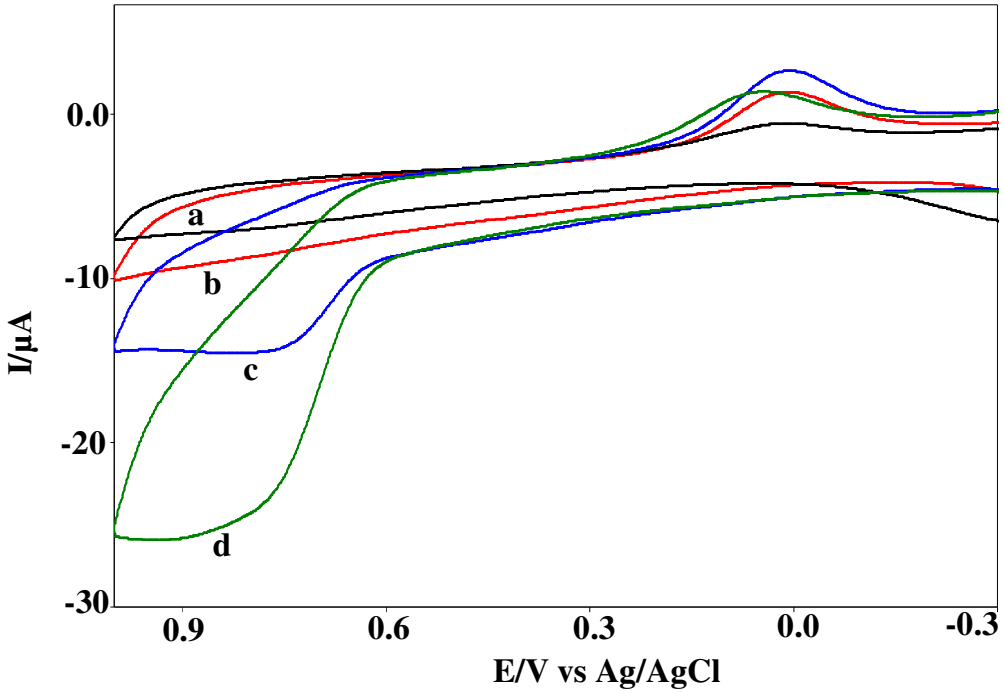

Fig. 4A Electrochemical responses obtained for the CuCP-SPCE and CuCP-ZnO-SPCE in the absence (curve a and b) and presence (curve c and d) of 100 µM NO solution at scan rate: 50 mV s^{-1} *vs.* Ag/AgCl.

Figure 4B

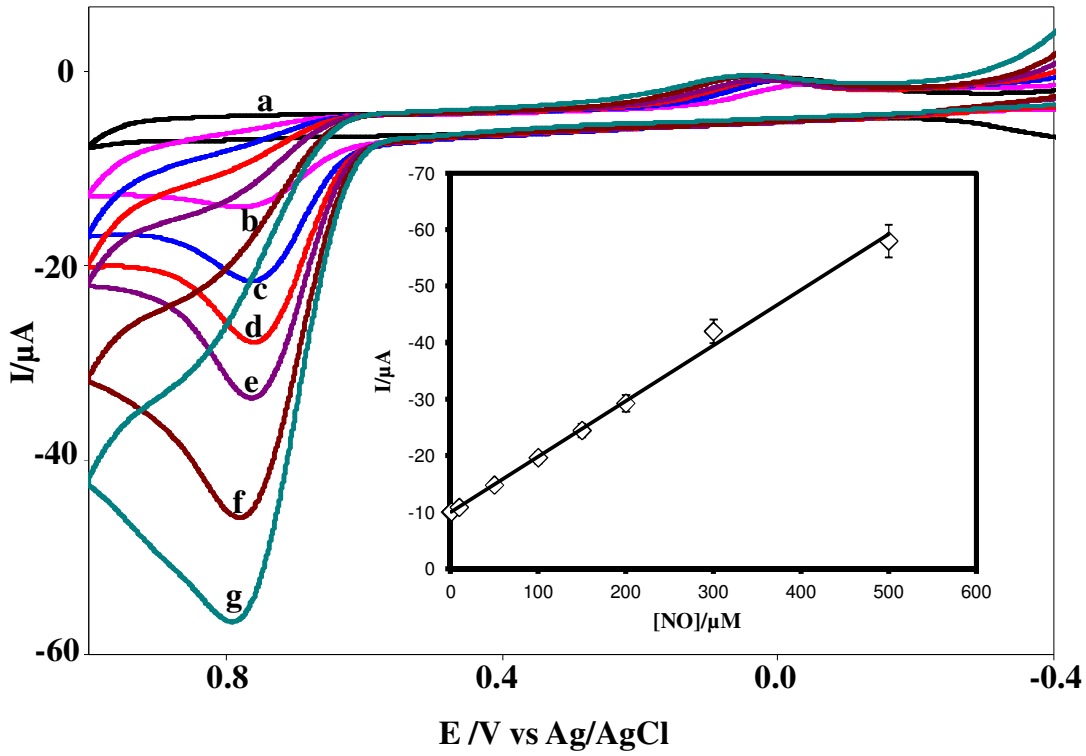

Fig. 4B. Electrochemical responses obtained for the CuCP-ZnO-SPCE in the presence of (a) control, (b) 50 µM, (c) 100 µM, (d) 200 µM, (e) 300 µM, (f) 400 µM and (g) 500 µM of NO solution at scan rate: 50 mVs^{-1} *vs.* Ag/AgCl. *Linear calibration curve (inset of Fig. 4B)* $y = -0.0983x-10.019$, $r^2 = 0.9968$.

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Figure 5A

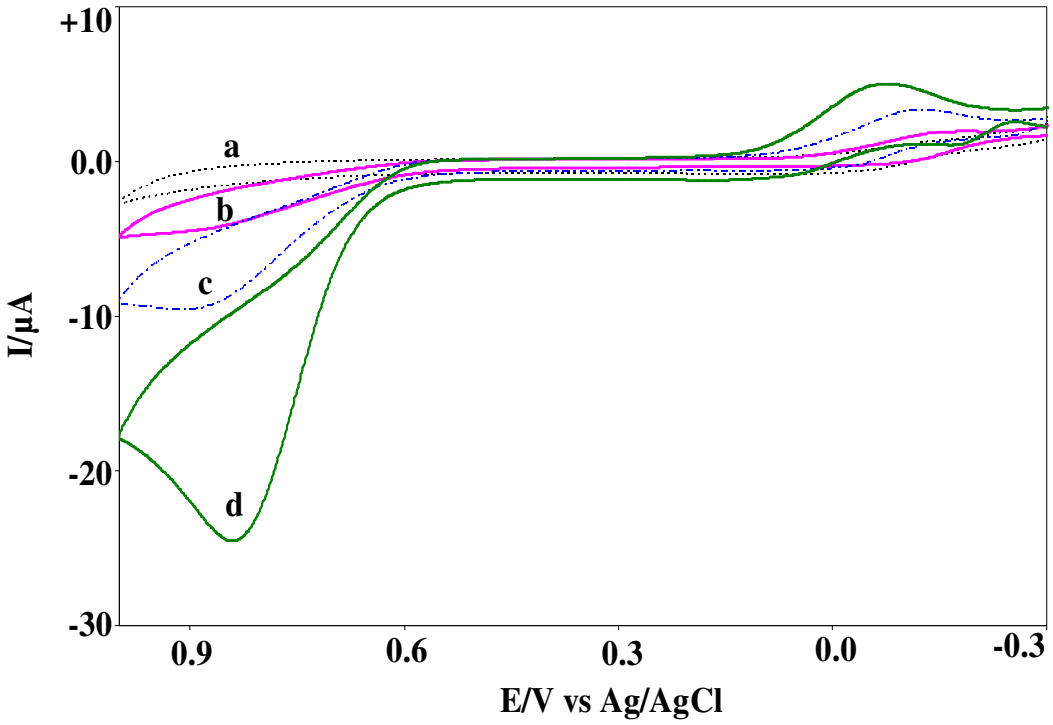

Fig. 5A . Electrochemical responses obtained for the CuCP-SPCE and CuCP-ZnO-SPCE in the absence (curve a and b) and presence (curve c and d) of 100 µM NO_2^- solution at scan rate: 50 mV s^{-1} *vs.* Ag/AgCl.

Figure 5B

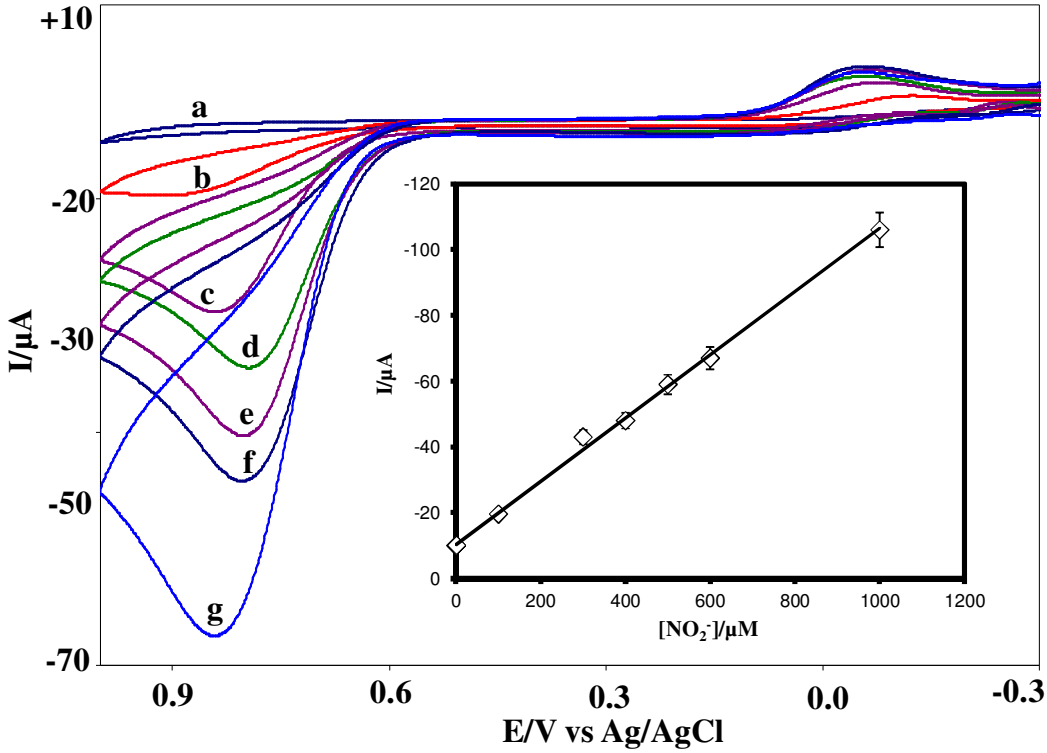

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Figure 6

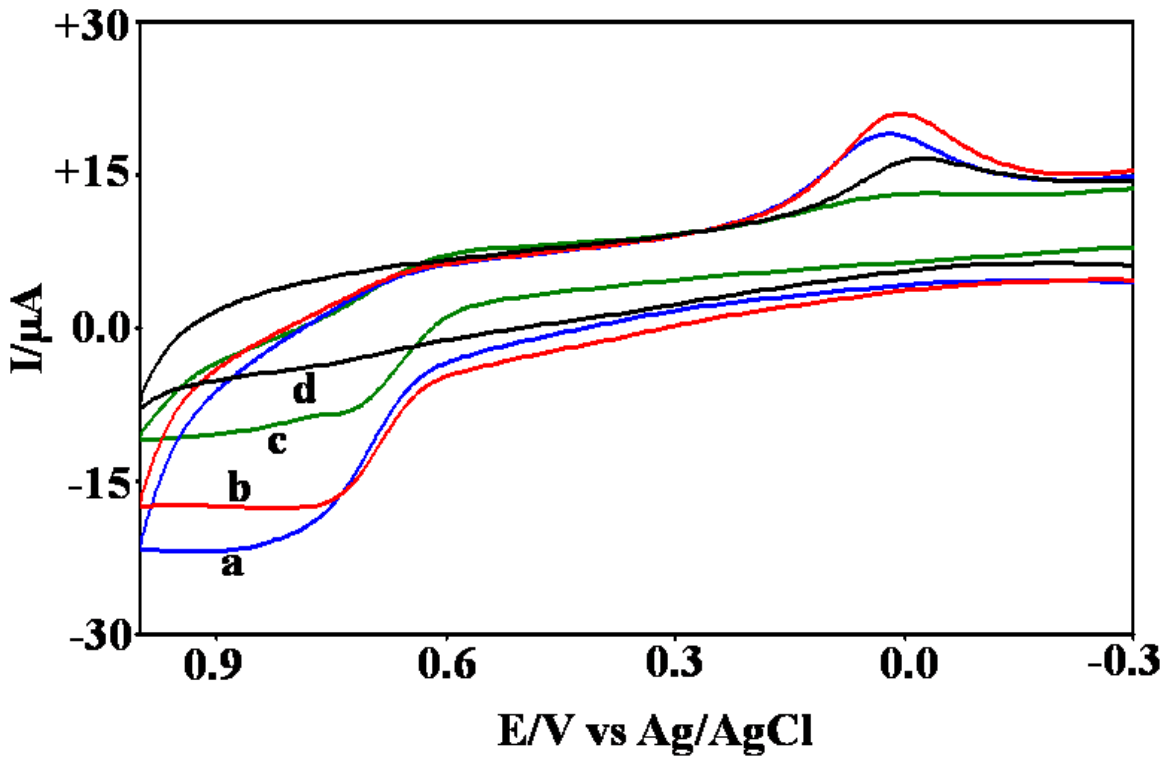

Fig. 6. Electrochemical responses obtained for the CuCP-ZnO-SPCE before and after nafion coating in (i) 0.1 M PBS containing 100 µM NO (curve b & c) and (ii) 0.1 M PBS containing 100 µM NO_2^- (curve a & d).

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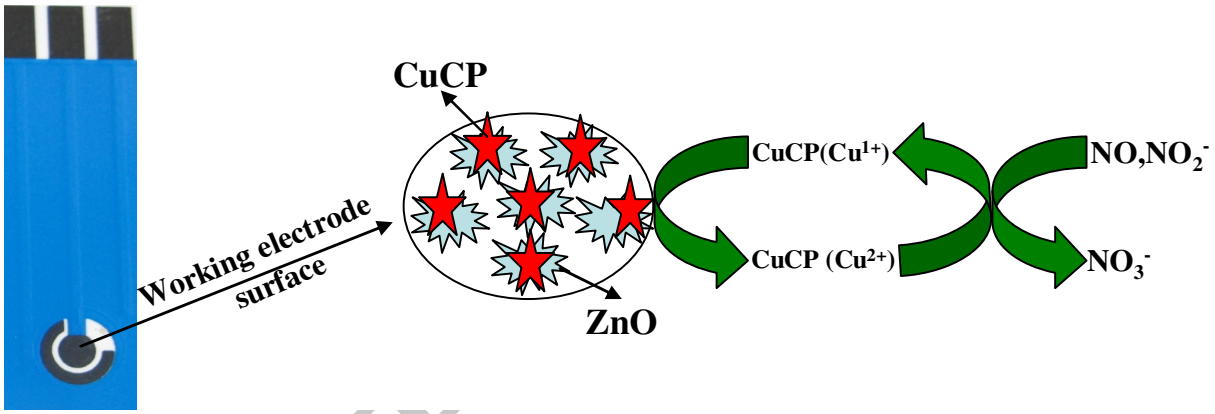
Scheme 1



Scheme 1. Schematic representation of the fabrication of CuCP-ZnO-SPCE and the illustration of the biochemical reaction occurring at the biosensors surface during the determination of NO, NO_2^-.

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Table 1: Electroanalytical properties of CuCP-ZnO-SPCE

Electroanalytical results	CuCP-ZnO-SPCE	
	NO	NO_2^- & NO_3^-
Linear range	200 nM - 500 µM	100 nM - 1 mM
Limit of quantification (LOQ)	100 nM	100 nM
Sensitivity	85.4 nA μM^{-1}	96.4 nA μM^{-1}
r^2	0.9942	0.9964
Stability	> 8 weeks	> 8 weeks
Accuracy (%error)	3	2
Slope ± error	-0.0983 ± 0.0006	-0.09866 ± 0.0003
Intercept ± error	-10.019 ± 0.178	-4.86 ± 0.1500

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Table 2: Electrochemical measurement of NO in human plasma

Individual	Concentration of NO (nM) ± SD
01	250 ± 12.0
02	260 ± 13.5
03	240 ± 10.4
04	245 ± 15.0
05	255 ± 14.3
06	250 ± 12.0

Electrochemical assay for determination of nitric oxide metabolites using copper(II)chlorophyllin modified screen printed electrodes.

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Table 3: Electrochemical measurement of total NO_2^- + NO_3^- levels in human plasma before and after beetroot supplementation

Individual	Total NO_2^- + NO_3^- Conc. by Griess method (µM)		Total NO_2^- + NO_3^- Conc. by Sensor (µM)	
	Before beet root juice	After beet root juice	Before beet root juice	After beet root juice
01	28.4 ± 1.8	29.7 ± 1.7	27.0 ± 0.9	28.8 ± 0.7
02	24.5 ± 1.1	32.1± 2.0	23.9 ± 1.3	31.7 ± 1.4
03	21.9 ± 1.3	26.1 ± 1.8	22.5 ± 0.8	24.8 ± 1.3
04	24.7 ± 1.4	28.9 ± 1.5	24.8 ± 1.1	29.2 ± 1.4