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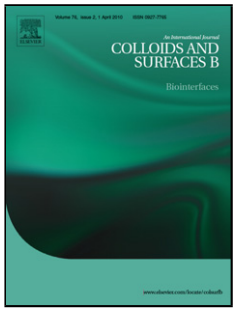

CuO nanorods as a laccase mimicking enzyme for highly sensitive colorimetric and electrochemical dual biosensor: Application in living cell epinephrine analysis

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CuO nanorods as a laccase mimicking enzyme for highly sensitive colorimetric and electrochemical dual biosensor: Application in living cell epinephrine analysis

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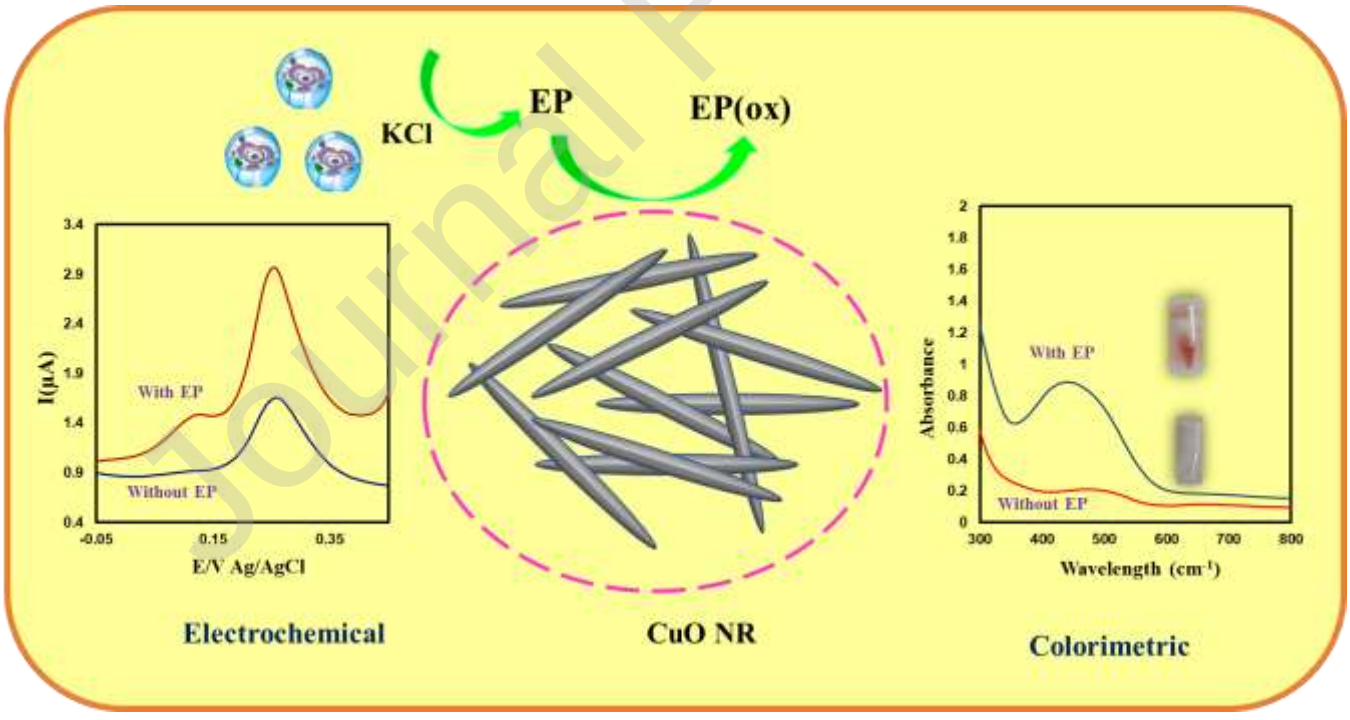
Graphical abstract



Highlights

- **CuO nanorods (NRs) with laccase like activity was synthesized via facile method in the room temperature.**
- **CuO NRs was used for colorimetric and electrochemical detection of epinephrine (EP).**
- **Best selectivity towards epinephrine in the presence of potential biological interferents.**
- **For the first time, the KCl-induced epinephrine secreted by live PC12 cells was detected by a CuO NRs modified GCE.**

Abstract

A sensitive colorimetric and electrochemical sensor for measuring of epinephrine (EP) was developed based on CuO nanorods (NRs), and applicability of the sensor for detection of release epinephrine (EP) from living cells was evaluated. The CuO NRs was prepared using a facile and efficient method in low temperature and characterized by Scanning electron microscopy (SEM), Transmission electron microscopy (TEM), X-ray powder diffraction (XRD), X-ray photoelectron spectroscopy (XPS), Fourier transform infrared spectroscopy (FTIR) and Energy-dispersive X-ray spectroscopy (EDX). The CuO NRs exhibited laccase-like activity and could oxidize epinephrine (EP) to a colored product. No interference from the common interfering agents such as dopamine, ascorbic acid and uric acid was observed. Colorimetric sensor demonstrated a linear range of 0.6-

18 μM with detection limit of 0.31 μM. Furthermore, the electrochemical study showed CuO NRs exhibited excellent electrocatalytic activity towards epinephrine oxidation. Differential pulse voltammetry signals increase with increasing of EP concentration in the range 0.04-14 μM, with a detection limit of 20 nM. Finally, the proposed sensor applied to perform real-time monitoring of epinephrine released by PC12 cells, indicating that CuO NRs provide a new platform for developing high-performance sensors in biological applications.

Keywords: CuO Nanorods, Laccase Mimicking enzyme, Epinephrine analysis, Colorimetric, Electrochemical, PC12 cells.

1. Introduction

Epinephrine (EP) is an important derivative of a neurotransmitter in the adrenal medulla and sympathetic nerve terminals. It plays an important role in the operation of the central nervous system (CNS), renal, hormonal and cardiovascular systems [1, 2]. The abnormal levels of EP led to many diseases, including phaeochromocytoma, hypoglycaemia, myocardial infarction, hypertension, sclerosis, Parkinson's and Alzheimer's disease [3-5]. People with Parkinson's and Alzheimer's disease have low epinephrine levels [6, 7]. Thus, the accurate detection and quantification of EP have a crucial role in better understanding and its essential functions in central nervous system for medical diagnosis and treatment [8]. To date, a great deal of methods such as liquid chromatography [9], polarography [10], capillary electrophoresis [11] chemiluminescence [12], colorimetric [13], fluorescence [14] and electrochemical techniques [15,

16], has been invested in the detection of EP. As regards epinephrine is easily oxidized, the detection techniques should have strong potential for rapid response, high sensitivity, easy-use, and low cost determination of EP [17].

Using enzyme-catalyzed oxidative reactions with specific catalytic activity for phenol compound would offer the possibility to the construction of rapid and sensitive sensor for detection of EP [18]. However, denaturation and high-cost purification of natural enzyme limited its applications. Thus, significant efforts have been made to develop materials that capable of mimic the catalytic activity of natural enzymes, known as artificial enzymes [6]. Notably, a variety of nanomaterials such as cobalt, cerium, nickel, copper and tungsten have been reported for use as artificial enzymes [19-21].

Most of the developed nanozymes are observed to exhibit peroxidase, oxidase or catalase-like activities. But the studies on other mimetic enzymes such as laccase are rarely carried out. It can attribute to the complex structure of the active site and catalytic mechanism of laccase. Laccases are a family of the multi-copper oxidases (MCOs), which catalyze the single-electron oxidation of the organic substrates, including polyphenols, *ortho*- and *para*-diphenols, aryl diamines, as well as polyamines [22, 23]. Furthermore, MCOs have been widely used in the fabrication of biocathodes and developments of biofuel cells.

Laccases have copper ions as the active center and exist in insects, bacteria and higher plants. They show potential in various applications such as dye bleaching, beverage stabilization, anticancer drug, water treatment and soil bioremediation. Nevertheless, poor enzyme stability, high cost and difficult recycling have seriously hindered the laccases application [24-27]. These problems could be addressed by construction of a nanozyme that mimics the natural laccase. Liang *et al*. prepared

laccase mimic nanozyme based on guanosine monophosphate (GMP) coordinated copper [28]. It showed excellent laccase-like activity for oxidation of phenol containing substrates such as hydroquinone, naphthol, catechol and epinephrine. The Cu/GMP nanozyme was more stable at extreme pH, high salt concentration and high temperature, compared to the protein laccase. Furthermore, Wang's group synthesized CH-Cu nanozyme with laccase-like activity [29]. The laccase-like activity inspired by the structure of the active site and the electron transfer pathway of laccase *via* the coordination of Cu^{+}/Cu^{2+} with a cysteine (Cys)-histidine (His) dipeptide. The degradation of phenolic pollutant and quantitative detection of epinephrine were established based on the CH-Cu nanozymes. Compared with noble metal nanocomposites, the copper nanoparticles are much cheaper. Therefore, much studies related to the preparation and application of copper-based nanoparticles was reported for the determination of other neurotransmitters such as dopamine [30-32].

In this investigation, we demonstrate the synthesis of CuO NRs with laccase-like activity and their application for the colorimetric and electrochemical determination of EP (Scheme 1). A deep structural and morphological characterization was performed by scanning electron microscopy, Transmission electron microscopy, X-ray powder diffraction, X-ray photoelectron spectroscopy Fourier transform infrared spectroscopy and Energy-dispersive X-ray spectroscopy. Both colorimetric and electrochemical biosensor showed high sensitivity and specific response toward EP compared to DA, AA and UA. The practicality of this fabricated biosensor was confirmed by determining the concentration of EP released from PC12 cells via exocytosis through stimulation of KCl. To the best of our knowledge, this work represents the first attempt of fabricating of the biomimetic based sensor for detection of EP in the living cell. The results demonstrated that the

presented biosensor could be used as an excellent tool for monitoring human health, especially those people who suffered from Alzheimer's and Parkinson's disease.

2. Experimental

2.1. Reagents and apparatus

Copper sulfate pentahydrate ($CuSO_4.5H_2O$), phytic acid ($C_6H_{18}O_{24}P_6$) and terephthalic acid ($C_8H_6O_4$) were purchased from Sigma Aldrich. Epinephrine (EP), dopamine (DA), ascorbic acid (AA) and uric acid was was obtained from Merck company. Phosphate buffer solution with pH 7 was used for all electrochemical measurements. All chemicals and reagents were of analytical grade with the highest purity and directly used without further purification. Deionized water from a Milli-Q Plus system (Millipore) was used in all solutions and experiment.

Scanning electron microscopy (SEM) and Transmission electron microscopy (TEM) image were obtained with a MIRA3 TESCAN HV: 20.0 kV from the Czech Republic and Philips microscope (EM 280, Tokyo, Japan), respectively. XRD patterns were recorded on a Bruker D8 Advance diffractometer equipped with a copper source and a general area detector diffraction system (GADDS). The FT-IR and UV–vis spectra were achieved by a Vector-22 Bruker spectrophotometer (Switzerland) and a SPECTROD 250-analytikjena spectrophotometer (Germany) respectively.). X-ray Photoelectron Spectroscopy (XPS) was recorded by Kratos AXIS Supra spectrometer using a monochromatic Al K(alpha) source (15mA, 15kV).

2.2. Synthesis of CuO NRs

The synthesis of CuO NRs was as follows: Firstly, $CuSO_4.{}_5H_2O$ (0.2 g) was dissolved into deionized water (10 ml). Secondly, 5 mL of phytic acid (6 mM) was dropped into the mixture solution. After stirring for 15 min, 5 ml of terephthalic acid (3 mM) was added to the above

solution. The system continued to react for 30 min. Then, the final product was washed with deionized water and dried at 40 °C for 12 h.

2.3. Colorimetric detection of EP

The catalytic ability of prepared CuO NRs was applied for colorimetric detection of EP. A 100 μL of epinephrine sample with different concentration was mixed with a catalyst (1 mg/mL, 300 μL). After 1 h reaction, the oxidation of epinephrine was monitored at 485 nm [28]. In the similar experiment, laccase was reacted with the same concentrations of epinephrine, and the result was compared.

2.4. Fabrication of CuO NRs modified GCE (CuO NRs /GCE) and electrochemical measurement

To fabricate the electrochemical sensor, at first, a bare GCE was successively polished with 1.0, 0.3, and 0.05 μm α-Al_2O_3 powder. Then, the prepared CuO NRs were dispersed in ethanol to give 1 mg/mL suspension. The modified electrode was obtained by dropping 5 μL of suspension onto the clean GCE surface and then evaporating the solvent in the environment. The electrochemical performance of CuO NRs /GCE was investigated using cyclic voltammetry (CV) and differential pulse voltammetry (DPV) with an Autolab electrochemical analyzer model PGSTAT30 (Eco Chemie, Utrecht, The Netherlands) driven with GPES software (Eco Chemie). In a three-electrode system, a modified CuO NRs/GCE, platinum wire and an Ag/AgCl electrode were used as the working electrode, the counter electrode and the reference electrode, respectively. The measurements were carried out in 0.1 M phosphate buffer solution (PBS) (pH = 7).

2.5 Cell culture and EP monitoring

We choose the PC12 cell line as a model for studying neurobiological functions and regulation of neurotransmitter release [33]. The PC12 cells were cultured in cell medium (DMEM),

supplemented with 10% fetal bovine serum, penicillin (100 U mL^{-1}), and streptomycin (100 mg mL^{-1}) in a humidified incubator at 37 ºC and 5% CO_2. Prior to the *in vitro* experiments, PC12 cells were removed from the bottom of cell culture flasks by standard trypsinization followed by centrifugation and suspended in the culture medium. To analyze EP using the CuO NRs /GCE modified electrode, DPV measurements were carried out by applying a potential between _0.1 V and 0.4 V vs Ag/AgCl. For the detection of EP released from living PC 12 cells, stimulus solution KCl (40-100 mM) was injected into the cells.

3. Results and discussion

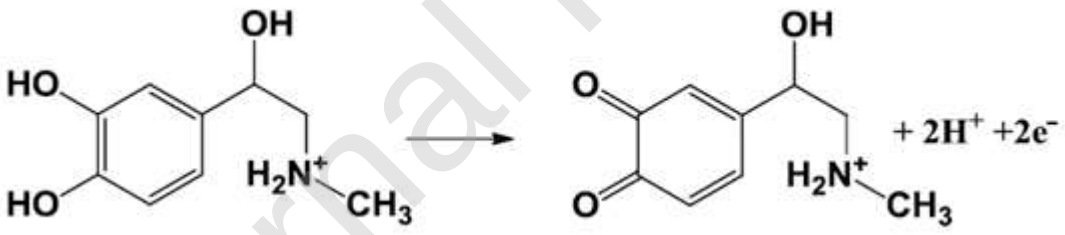
3.1 Morphology and Characterization of CuO nanocomposite

The typical morphologies of CuO nanocomposite were observed by SEM and TEM, are presented in Fig. 1, A, B. As can be seen, the prepared sample is mainly composed of relatively uniform nanorods with vermiform-like structures. From a high magnification TEM image in Fig. 1. B it is clearly observed that all nanorods have smooth surfaces with average the diameter of 15 nm. The EDS analysis of the as-prepared CuO NRs in Fig. 2. B revealed its elemental composition containing copper. As well as, FTIR spectroscopy was conducted to investigate the compositions of the prepared sample. As can be seen from the spectra of phytic acid in Fig. 2. D, the peaks at 1679 cm^{-1} is assigned to the stretching vibration of the P=O bond, and the peaks at 1208 and 908 cm^{-1} ascribed to symmetric and asymmetric vibration of P-O-C (curve a). The characteristic peaks of phytic acid were decreased and shifted after adding Cu^{2+} (curve b), indicating that the phosphate group of phytic acid are involved in Cu^{2+} binding. The XRD patterns of the synthesized nanorods are shown in Fig. 2. A. The observed diffraction peaks are indexed to the diffraction of CuO with JCPDS card no. 89-2530 [34].

The high-resolution XPS spectrum of Cu was shown in Fig. 2. C. As can be seen the Cu $2p_{3/2}$ peaks at 934.8 eV with a satellite peaks at 943.4 eV and Cu $2p_{1/2}$ peaks at 954.6 eV with a satellite peak at 962.7 eV, was detected, respectively. The well-defined satellite peaks in XPS indicates the presence of the Cu^{2+} species. These features indicative Cu^{2+} state for the Cu atoms [35-37].

3.2 Electrocatalytic activity of CuO NRs

The electrocatalytic activity of CuO NRs was investigated by modifying GC working electrode with the CuO NRs. Cyclic voltammetry, recorded for (a) bare GCE, (b) CuO NRs/GCE, (c) bare GCE in 1 µM EP, and (d) CuO NRs/GCE in 1mM EP (Fig. 3. A). The experiment was conducted in 0.1M PBS (pH 7) with a scan rate of 50 mV s^{-1}. There are no peaks in the CV curves for bare GCE in PBS without EP (green line). Upon EP introduction to the bare GCE, an oxidation peak was observed at 0.34 V (red line), corresponding to the oxidation of epinephrine to epinephrinequinone according to following reaction [38] (eq. 1).



(eq. 1)

Epinephrine **Epinephrinequinone**

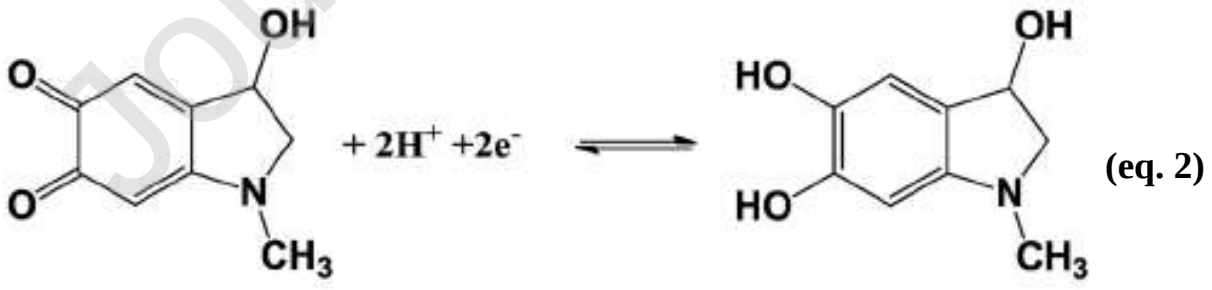
The CuO NRs modified electrode exhibits a pair of redox peaks with the anodic and cathodic peaks at about 0.12 and -0.18 V, respectively (block line), which are assigned to Cu(0)/Cu(II) redox couple [39]. After addition of EP, the analytical signal of CuO NRs was noticeably increased in the oxidation and reduction potentials (purple line). It is noteworthy that the oxidation peak of EP was shifted negatively, as well as the reduction peak corresponding to the electrochemical

reduction of epinephrinequinone to epinephrine could be observed. The above results confirm that the CuO NRs have a great electrocatalytic activity toward EP detection.

To explore the chemical reason for catalysis, CuO was prepared without phytic acid or terephthalic acid. In the absence of terephthalic acid, no precipitant was produced, and the sample without phytic acid shows no response toward EP (Fig. 3. B). Therefore, the interaction between Cu^{2+} and phytic acid is required for the activity, while the terephthalic acid only provides coordination sites.

3.3. Sensing Properties of the CuO NRs

As an analytical application, the prepared CuO NRs was evaluated for a dual electrochemical and colorimetric detection of epinephrine. The electrochemical sensing properties of CuO NRs modified electrodes were investigated by recording of differential pulse voltammograms of EP solutions. As shown in Fig. 4 A, the oxidation peak currents increased with increasing concentrations of EP from 0.04-14 μM. The oxidation peak at 0.25 V is related to the oxidation of epinephrine to epinephrinequinone. During a series of chemical reactions, epinephrinequinone loses a proton and forms adrenochrome. Thus, an oxidation peak was at 0.12 V, can be attributed to oxidation of leucoadrenochrome to adrenochrome based on a well-established mechanism [40] (eq. 2).



(eq. 2)

Adrenochrome **Leucoadrenochrome**

The calibration curve showed good linearity (Fig. 4. B), with a correlation coefficient of 0.9956 and a detection limit of 20 nM. Further, the sensing performance of CuO NRs toward EP measuring was also studied by colorimetric method. It was found that upon addition of EP to the catalyst, the colorless solution of EP was changed to light brown-red color. The colored oxidation product of this reaction could be used for epinephrine sensing. So, in addition to visual analysis, the sensitivity of the assay was further monitored by UV-vis spectroscopy (Fig. 4. C). As illustrated with increasing the concentration of EP, the absorbance at 485 nm is increased. The plot in Fig. 4. D displayed a good linear relationship with the concentration of EP in the range of 0.6-18 μM. The detection limit was calculated as 0.31 μM. Both electrochemical and colorimetric methods possess a broad linear range and a low detection limit. The analytical performance of the proposed sensor for detection of EP was compared with some examples of EP sensors in literature as presented in Table 1. As can be seen, the analytical properties of the proposed laccase sensor are comparable or better than reported results.

An abreast comparison was made between CuO mimicking laccase and the natural laccase. As shown, the higher activity was observed with the same concentration of EP for CuO mimicking laccase compared to natural laccase (Fig. 5. A). These results indicate the acceptable bioactivity of CuO NRs as mimicking laccase enzyme toward EP oxidation.

3.4 Selectivity and anti-interference study

Selectivity is a key factor to evaluate the properties of the sensors, especially for the sensor with potential applications in practical biological samples. In order to investigate the selectivity of the laccase mimic for EP, the response of CuO NRs to different interfering species containing AA,

UA and DA as the structural analogues or coexisted compounds, was tested. The concentration of interferences is ten times higher than the EP. The both current and absorbance response of EP or each interference was shown in Fig. 3. C, D. It can be seen that the DPV response of these interfering compounds is negligible. As well as, the maximum absorbance UV-spectroscopy in 485 nm is obtained after the presence of epinephrine in solution. These results demonstrate the high selectivity of both colorimetric and electrochemical measuring methods for EP detection and validate the idea that the proposed probe can be applied for selective dual-mode detection of EP.

3.5. Detection of EP released from live cells

The above results clearly showed the high performance of CuO NRs mimicking laccase toward EP detection, making it highly promising for following EP released from living cells. Clearly, monitoring of cell-released EP is beneficial in deeply understanding of the central nervous system and early diagnosis of chronic neurodegenerative disorders. PC12 cells are chosen as a neurons cell model to study the performance of the CuO NRs for sensing endogenous EP.

In order to detection of EP released from the PC 12 cells, KCl solution was injected subsequently as a stimulus. Fig. 5. B shows EP release experimental results which were recorded by DPV versus stimulation of in different concentrations of KCl solution (40-100 mM). As can be seen, the current intensity increased in a concentration dependent manner. This observation clearly revealed that the CuO NRs have great potential for live cell neurotransmitter signaling.

4. Conclusion

In summary, a facile and efficient method in low temperature was used for the synthesis of CuNRs and its application as a probe with laccase mimicking activity for electrochemical and colorimetric detection of EP. This probe with dual recognition capability showed high sensitivity and excellent

selectivity. A good linear relationship is obtained from 0.6-18 μM and 0.04-14 μM for EP colorimetric and electrochemical detection, respectively. More significantly, the established nanosensor used for real-time detection of EP released from PC12 cells. It was the first report on EP exocytosis monitoring, which can be used for the observation of neurotransmitter related intracellular signal transduction events and clinical diagnostics. The proposed strategy may offer in depth insight into disease related molecular targets.

Credit

Negar Alizadeh: Conceptualization, Writing - Original Draft, Writing - Review & Editing.
Shadi Ghasemi: Investigation, Data Curation.
Abdollah Salimi: Supervision, Project administration, Funding acquisition.
Tsun-Kong Sham: Supervision.
Rahman Hallaj: Supervision.

Decleration

The authors declare that they have no conflict of interest to the publication of this article.

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Figure captions

Scheme. 1. (A) Schematic representation of electrochemical and colorimetric EP detection on CuO NRs laccase enzyme mimic.

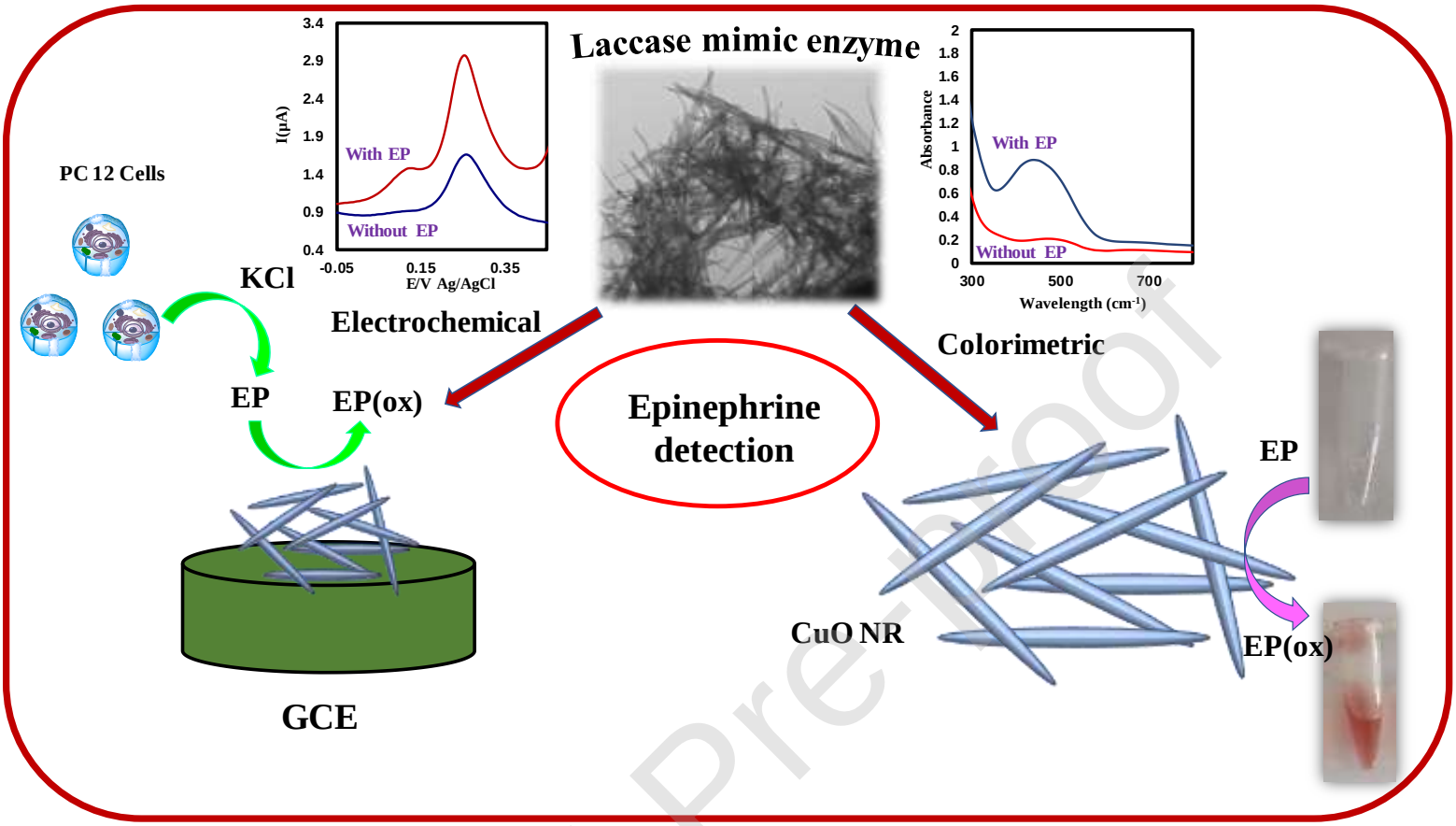
Fig. 1. (A) SEM image and (B) TEM image of CuO NRs.

Fig. 2. (A) XRD patterns, (B) EDS analysis (C) Cu 2p XPS spectra of CuO NRs and (D) FTIR spectra of (a) phytic acid (b) CuO NRs.

Fig. 3. (A, B) Cyclic voltammogrames of different electrodes in 0.1 M PBS (pH=7) with scan rate of 50 mV s^{-1}, selectivity of (C) colorimetric and (D) electrochemical sensing system towards EP and other interferences.

Fig. 4. (A) DPV and (C) absorbance responses of CuO NRs upon addition of various concentrations of EP, the linear calibration plot of (B) current and (D) absorbance versus the concentration.

Fig. 5. (A) Absorbance responses of laccase upon addition of various concentrations of EP (B) DPV responses of CuO NRs/GCE to EP released from PC12 cells with the stimulant of KCl in a 0.1 M PBS solution.



Scheme. 1

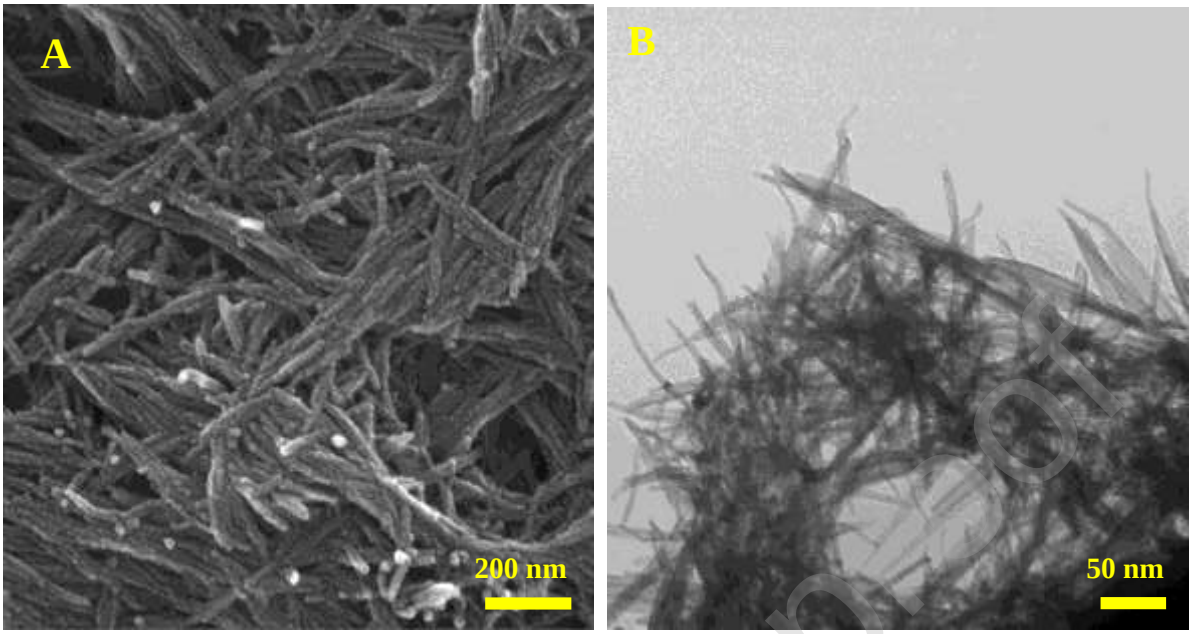


Fig. 1

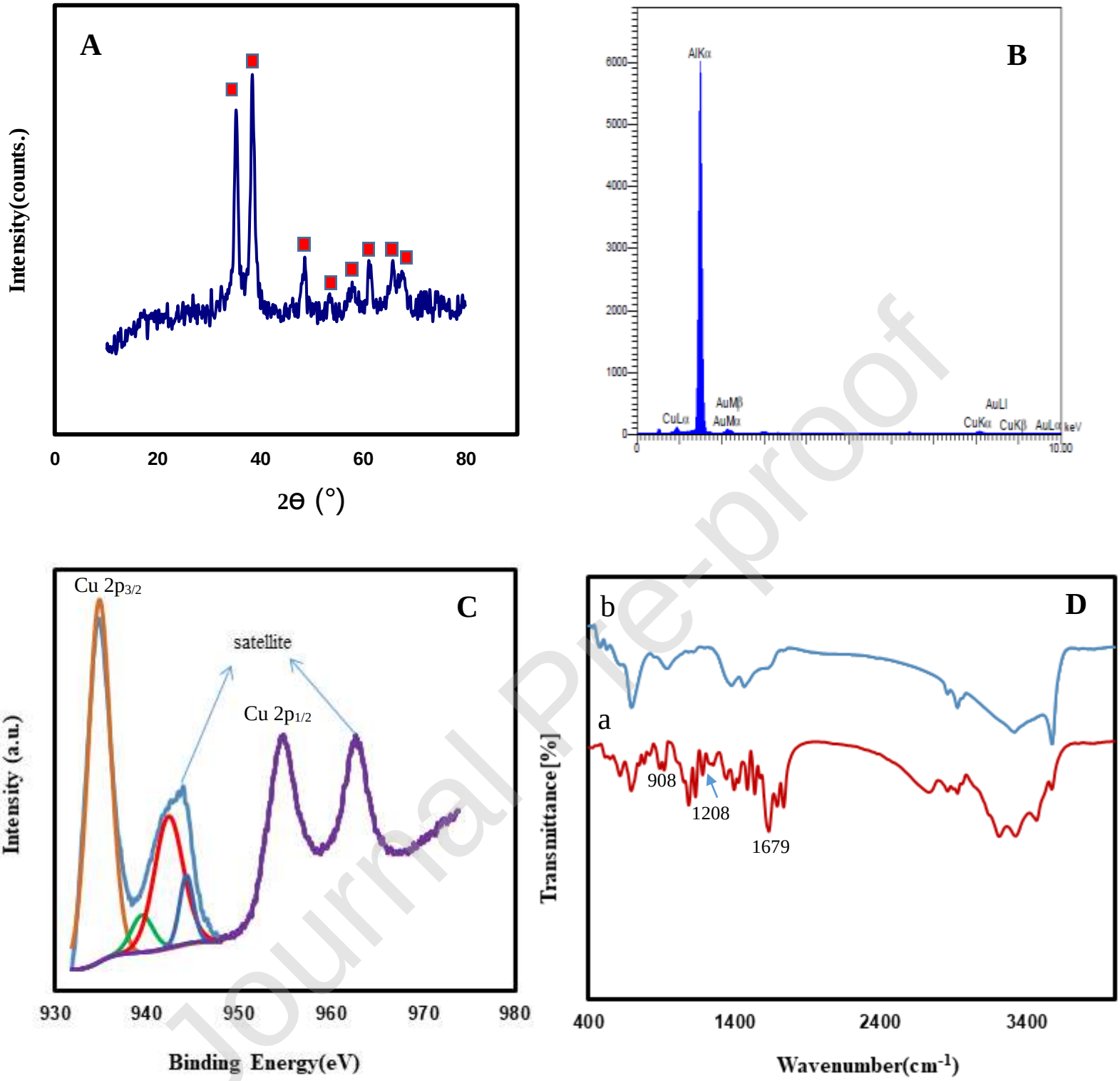


Fig. 2

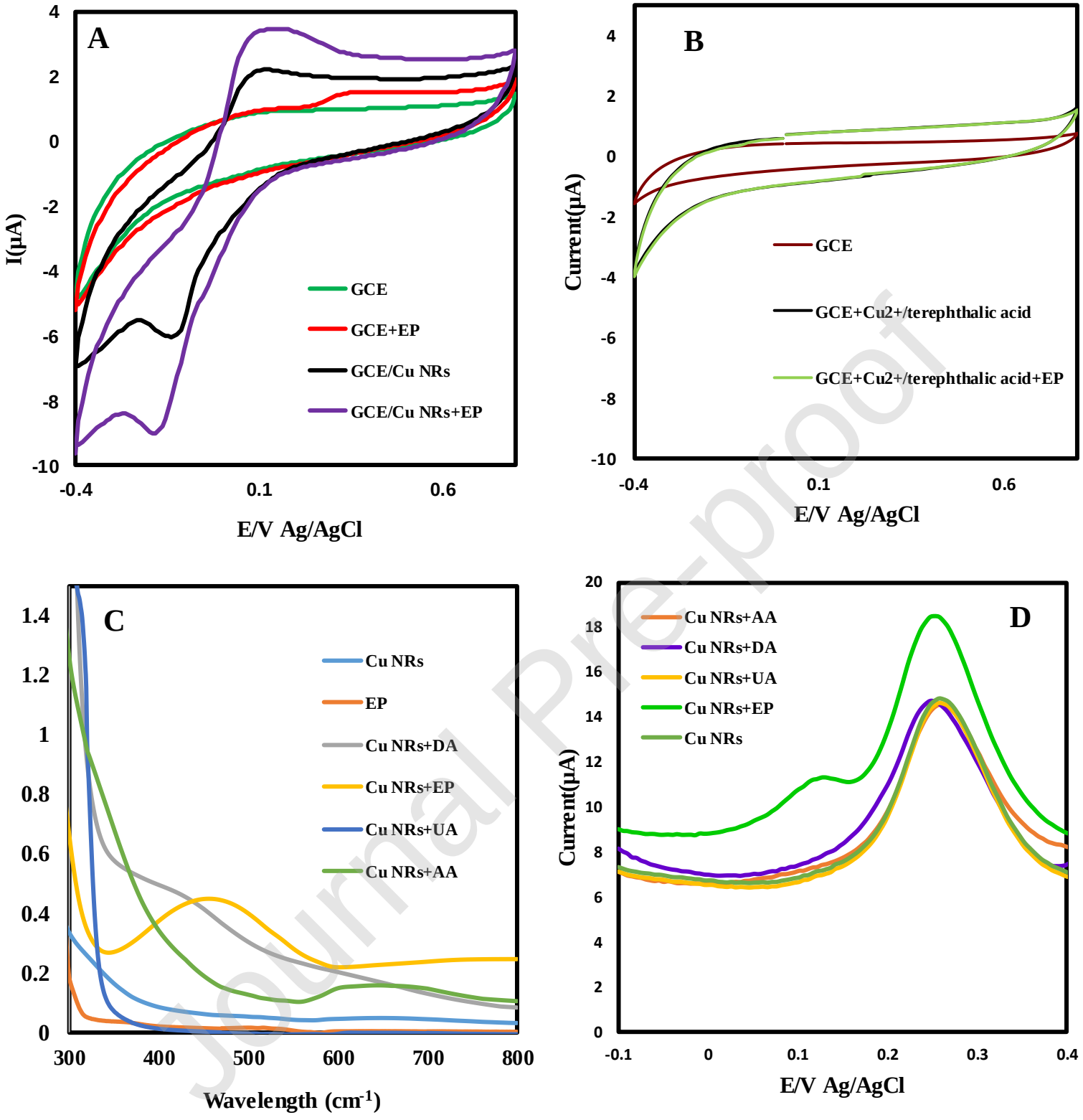


Fig. 3

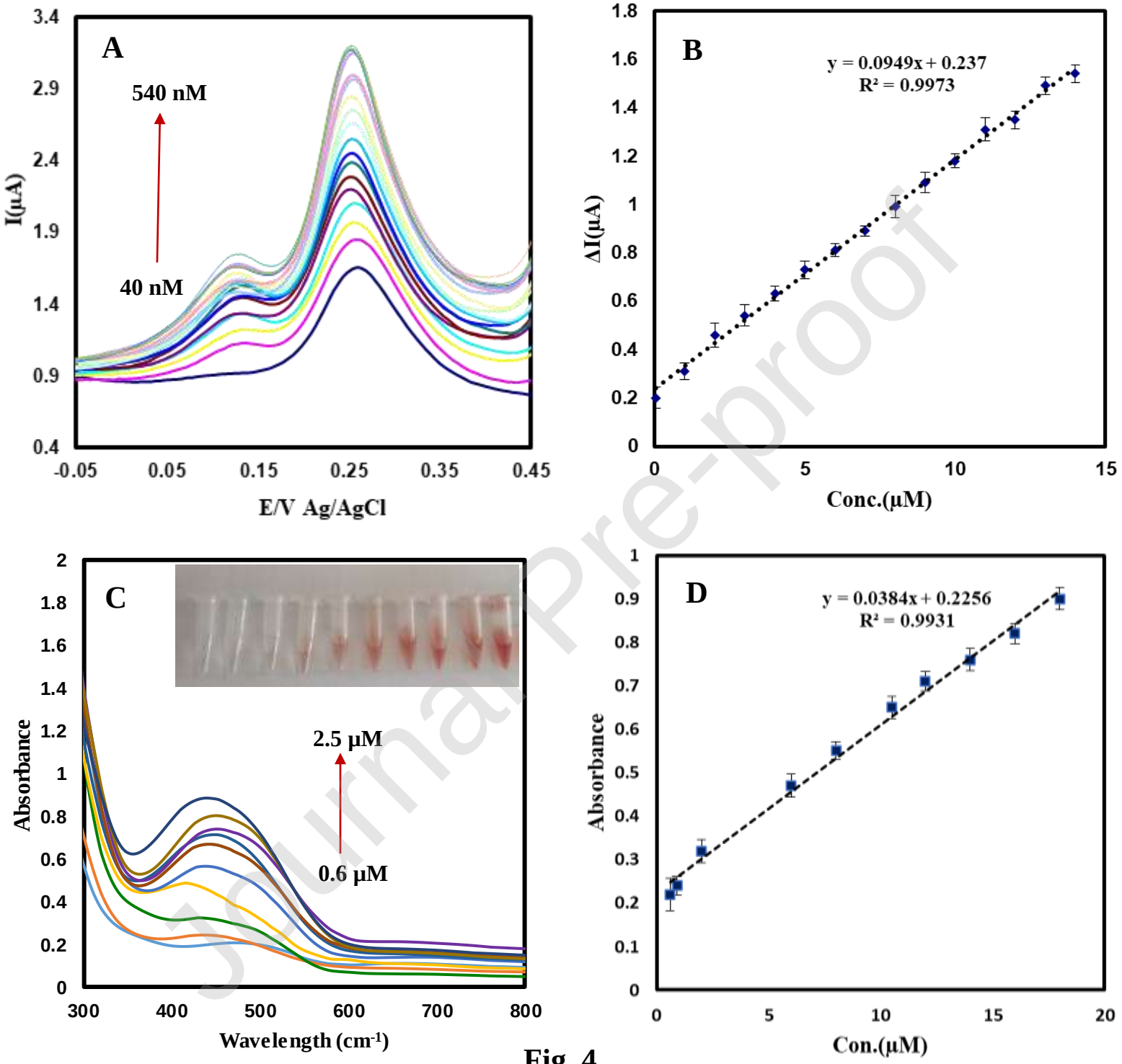


Fig. 4

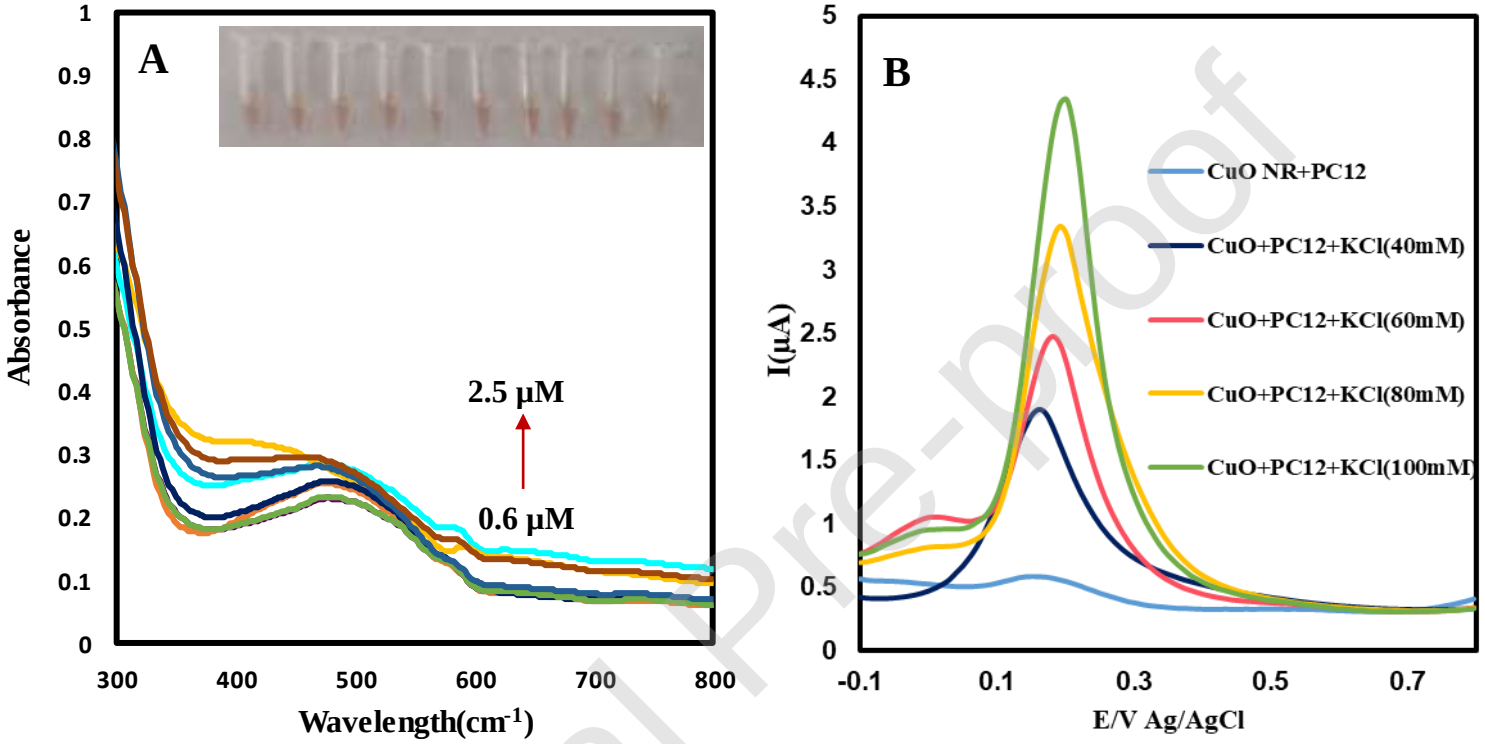


Fig. 5

Catalyst	Linear range (µM)	Detection limit (µM)	Method	Reference
SnO_2-Graphene	0.5-200	0.017	Electrochemical	[41]
Au nanotube	10-150	2.8	Electrochemical	[4]
Au 4Mpy AuNPs	10-60	4.5	Electrochemical	[42]
PMB/MWCNT	2.5–800	69.9	Electrochemical	[43]
f-MWCNTs/BR9	19.6–82.5	9	Electrochemical	[44]
Poly(taurine)	2-600	0.3	Electrochemical	[45]
EDDPT/GO	1.5–600.0	0.65	Electrochemical	[2]
Au-MWCNTPANI-RuO_2	4.9- 76.9	0.18	Electrochemical	[46]
Caffeic acid	2.0–80	0.6	Electrochemical	[47]
Au–Ag	25–700	5.05	Electrochemical	[48]
Pt@SnO_2	13.75–110	0.35	Electrochemical	[49]
MWCNT	22.5–547	3.92	Electrochemical	[50]
CuO NRs	0.6-18	0.31	Colorimetric	This work
CuO NRs	0.04-14	0.02	Electrochemical	This work

Table 1. Comparison of the response characteristics of CuO NRs with other EP sensors.