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CHARACTERIZATION OF CERIUM OXIDE-CHITOSAN NANOCOMPOSITE–MODIFIED SCREEN PRINTED CARBON ELECTRODE AND APPLICATION IN MELATONIN DETERMINATION

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ABSTRACT: A simple and effective modification of the working screen printed carbon electrode (SPCE) surface has been developed with a mixture of chitosan biopolymer and nano cerium oxide at 1:1 composition for the detection of melatonin, a circadian rhythm regulation hormone. The fabricated electrode was characterized by scanning electron microscopy (SEM), energy dispersive X-ray spectrophotometer (EDS), electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV). Electro-oxidation behaviors of melatonin were extensively observed by differential pulse voltammetric(DPV) analysis. The experimental results demonstrated that the modified cerium oxide-chitosan screen printed carbon electrode (SPCE-Chi-CeO_2) exhibited satisfactorily improvement in the electrochemical current sensitivity.Alinear calibration curve for melatonin was obtained in the concentration range from 0-10 µg/ml with a detection limit of 0.18 µg/ml based on DPV measurement. Good selectivity was also obtained in an interference study with some other closely structural related compounds such as tryptophan and serotonin. Thus this modification strategy was proved to be a suitable tool for the simple, fast, sensitive and selective determination of melatonin.

Keywords: SPCE-Chi-CeO_2, SEM and EDS, EIS and CV, Melatonin, DPV analysis

1. INTRODUCTION

Melatonin (N-acetyl-5-methoxytryptamine) is an important hormone that is synthesized in the pineal gland.It is also recognized as ubiquitous among living organisms, including humans, animals, plants, bacteria, fungi, and algae [1]. It is responsible for regulating sleep and circadian rhythms in mammals, and is used to counteract the effects of jet-lag or to aid sleep. Principally, it is used for the diagnosis and remedy of insomnia, hypomnesia and of the cerebrum and is used to treat some diseases such as Alzheimer and cancer. Melatonin is biomarker of circadian dysregulation, and measurement melatonin levels in saliva and blood, and the melatonin metabolite 6-sulphatoxymelatonin in urine are useful to evaluate problems related to circadian rhythms and sleep. The development of rapid, simple, easy to use, real-time detection, low cost and portable detection of melatonin in animal tissues and fluids emerges as imperative. The biosensor is methodologies employed for qualitative and quantification detection of melatonin in cells, animals and human products.

Although many methods have been applied in the qualitative and quantitative determination of low levels of melatonin in samples such as enzyme linked immunosorbent assay (ELISA) and radioimmunoassay (RIA),high-performance liquid chromatography (HPLC) and capillary electrophoresis; but there are expensive and time consuming methods and are confined to the laboratory. ELISA and RIA provide a stable calibration curve, reasonable speed and small sample volume with specificity, sensitivity and precision [2], but they have some limitations and disadvantages by their high cost and laborious time requirement. Moreover, a special lab in radiochemistry is needed in RIA analysis and the radioactive tracer is toxic as well. Recently, electrochemical biosensors have expanded their attractive attributions that offer new challenges of analytical methodology and a possibility of miniaturization and portability, sensitivity, selectivity, a wide linear range, energy saving, minimal space, and cost effective usage.

Screen printed carbon electrode (SPCE) in combination with suitable surface modification and a proper electrochemical technique has been shown applicable in various electro-catalytic studies. Previous works on the fabrication of an effective and affordable electrochemical implement via a route of this SPCE modification have provided a possibility for the development of a potent biosensor [3]. The versatile chitosan biomaterial has been employed as main structural scaffold for a wide variety of biosensor due to its robust properties especially an ionic binding of biomolecules including DNA and proteins [3, 4,

5].In addition, many types of nanoparticles such as metals and metal oxides have provided an ideal remedy for enhancing the sensing performance of the sensors. Cerium oxide (CeO_2) is one among the metal oxides mostly introduced for biosensor fabrication due to its excellent properties, including a good biocompatibility, high chemical stability, excellent electronic conductivity and huge electroactive surface. A composite matrix construction based on chitosan and CeO_2 for an immobilization of single stranded DNA probehas also been reported for effective sensing of cancer gene [6].

In the present study, modification of SPCE with a combination of chitosan and CeO_2 is organized to fabricate an electrochemical sensor for melatonin determination via differential pulse voltammetry (DPV). Techniques in scanning electron microscopy (SEM), energy dispersive X-ray spectrophotometer (EDS), electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) are utilized to investigate the modified SPCE performance. An appreciable sensitivity of SPCE-Chi-CeO_2 in the detection of melatonin is expected from the inheritance of advantageous chitosan and CeO_2 properties as well.

2. MATERIALS AND METHODS

2.1 Materials and instruments

Chitosan (85% degree of deacetylation) with a molecular weight of 0.28 kDa was obtained from Bioline Lab, Co., Thailand. Cerium oxide (CeO_2) nanoparticles (50 nm), tryptophan and serotonin were gained from Sigma-Aldrich Co. LLC, USA. Melatonin (GMP 99.96%) was acquired from Huanggang Saikang Pharmaceutical Co., Ltd, Hubal, China. Other chemicals for reagent preparation were purchased from Ajax Finechem Pty, Ltd., Australia. Buffer solutions and reagents were made up using organic free ultrapure water from an Elga DV25 Pure water OptionQ system with resistivity (25 °C) >18.18 MΩ•cm.Screen printed electrode DS-150 strip made of carbon as working area (4 mm diameter) with a counter electrode made of platinum, a reference electrode made of silver and an electric contact made of silver was provided by DropSens, ParqueTecnológico de Asturias, S.L. Llanera (Asturias) Spain. A scanning electron microscope (SEM) LEO1450VP was from Hurley, UK and anenergy dispersive X-ray spectrophotometer (EDS) was from Technai G2 20,FEI; Thermo Fisher Scientific, Oregon, USA. Electrochemical performances were acquired with a potentiostat/gulvanostat model PGSTAT 302N of ECoChemie Autolab (MetrohmAutolab B.V., Utrecht, The Netherlands) incorporated with Nova system software.

2.2 Modification of working electrodes

Fabrication of the modified SPCE-Chi-CeO_2 electrode was done using a DS-150 screen printed carbon electrode (SPCE) as the basal platform. The 0.25 cm^2 working surface area of the SPCE was cleaned by ultrapure water rinsing for 3 times prior to a modification. Chitosan solution was made by a mixing of chitosan powder with 1% acetic acid at 1% by weight per volume. Afterward, the nano-cerium oxide (CeO_2) particles were added up at 1 % (w/v) and sonicated for 15 min at room temperature. Later, a 5 µL of this Chitosan-CeO_2 solution was embedded onto the 0.25 cm^2 SPCE working surface and left drying in clean air environment overnight. Then it was rinsed with ultrapure water and allowed to dry again for the ready use.

2.3 Structural and electrochemical characterization

Surface morphological characteristics of the modified SPCE-Chi-CeO_2 in comparison to its basal SPCE were acquired through scanning electron microscopy (SEM).Three replicates of each were sputter coated with gold and 0.25 cm^2 working electrode areas were investigated using 10,000×magnifications. A structural profiling of the elements was consequently investigated by energy dispersive X-ray spectrophotometer (EDS) in couple with the SEM. Electrochemical performance were done via Autolab PGSTAT 302N controlled with the Autolab Nova software. An electrolyte 0.1 M phosphate buffer saline (PBS) at pH 7.0 solution containing 1.0 mM of $K_3Fe(CN)_6$as a model electrochemical probe was employed for the electrochemical characterization. Electrochemical impedance spectroscopy (EIS) was carried out by applying a potentiostatic state of 0.01 V AC potential and 0.17 V bias potential with 10 mV amplitude in the frequency range from 0.01 to 10^5 Hz. Cyclic voltammograms were obtained over the potential scan range from -0.6 to 1.2 V at a varied scan rate of 25, 36,81,144 and 255 mV/s. Subsequent measurement of electrochemical response of SPCE-Chi-CeO_2 to a function of melatonin concentration (0-10 µg/ml) was set up using differential pulse voltammetric (DPV) technique at 50 mV/s scan rate.

3. RESULTS AND DISCUSSION

3.1 Morphological and structural characterization of modified SPCE-Chi-CeO_2

The surface morphologies and atomic components of SPCE-Chi-CeO_2 in comparison to the basal SPCE at different mixing molar percentage were characterized by SEM and EDS. The SEM image in Fig. 1A presents homogeneous carbon platelets containing in SPCE working surface, whereas the modified SPCE-Chi-CeO_2 surface has uniform granular porous morphology attributed to the homogeneous dispersion of CeO_2 nanoparticles in chitosan network as seen in Fig. 1B. Seemingly, a stable surface cover could be obtained following the modification by this highly adhesive nanocomposite. Fig. 2 represents atomic fingerprints from EDS investigation that signify the differences between SPCE (Fig. 2A) and SPCE-Chi-CeO_2 (Fig.2 B). The bare SPCE has shown its dominant containing elements as carbon, chlorine and oxygen. The carbon is presented as the major constituent of SPCE, and chlorine is present as a constituent of the binder or solvent, whereas the presence of oxygen is due to any hydrophobic contamination.

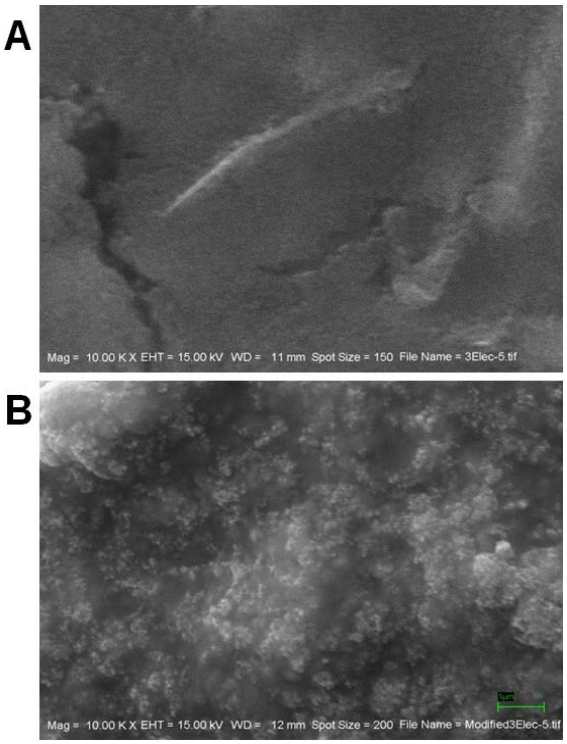


Fig. 1 SEM images observed from working surface of bare SPCE (A) and SPCE-Chi-CeO_2 (B).

In view of the modified SPCE-Chi-CeO_2, many more types of atoms are presented such as fluorine, nickel, sodium, technetium, gadolinium and gold as well as cerium component in Chi-CeO_2 matrix. The carbon in this case is assumed as a constituent of the chitosan and electrode, the presence of oxygen is due to any hydrophobic contamination and from chitosan, fluorine, nickel, sodium and technetium maybe from electrode plastic basement and contamination, while gold is from gold coating electrode for EDS in couple with SEM analysis. The percentage of atomic and weight by element and total compound in SPCE-Chi-CeO_2 are as displayed in Table 1. The atomic percentage of carbon is present as 23.68% in chitosan constituent and 33.92% in the electrode basement. While the atomic percentage of CeO_2 molecules and total chitosan molecules are 20.42 and 3.95, respectively. Considering the weight percentage by compound, it has been stated that the carbon content is about 8.5%, the CeO_2 molecules are 73.35% and total chitosan molecules are 12.77%. Thus, it can be concluded that in SPCE-Chi-CeO_2 the major constituent by atomic percentage is carbon and by weight percentage is cerium.

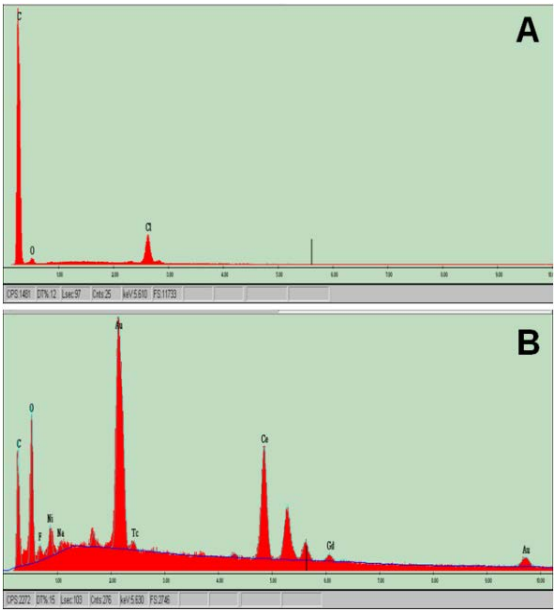


Fig. 2 Atomicfingerprints ofbare SPCE (A) and SPCE-Chi-CeO_2 (B) components by EDS analysis.

3.2 Electrochemical characterization of modified SPCE-Chi-CeO_2

EIS analysis has provided information about the changes on interface properties between the basal SPCE and the modified SPCE-Chi-CeO_2 as illustrated by Nyquist plots in Fig. 3. The semicircle diameter represents the electron charge-transfer resistance (*Rct*) character which controls the electron transfer kinetics of the redox probe ($K_3Fe(CN)_6^{3-/4-}$ in this experiment) at the electrode interface [7].

Table 1 Percentage of atomic and weight by element and total compound in SPCE-Chi-CeO_2 from EDS analysis.

Components	Atom (%)	Weight(%)
Elements		
C	57.60	-
O	29.40	19.82
F	2.27	1.81
Ni	2.42	5.99
Na	1.09	1.06
Tc	0.37	1.54
Ce	6.81	40.18
Gd	0.07	0.47
Compounds		
Total chitosan molecules	3.95	12.77
CeO_2 molecules	20.42	73.35
C in chitosan	23.68	-
C in SPCE basement	33.92	8.50

The bare SPCE showed a large semicircle diameter with *Rct* value about 8,000 Ω/cm^2 (Fig.3 curve a), whereas the modified SPCE-Chi-CeO_2 produced almost half size smaller with the *Rct* value down to about 3,000Ω/cm^2 (Fig.3 curve b) implying less charge transfer resistance and hence about 2.7 times better electron transfer ability. An extreme faster in electron transfer obtained following the modification of SPCE with CeO_2-chitosan nanocomposite probably due to the robust positive charges containing in this composite that offer electrostatic anchors for negative charges of the probe to accelerate the electron transfer at the interface.

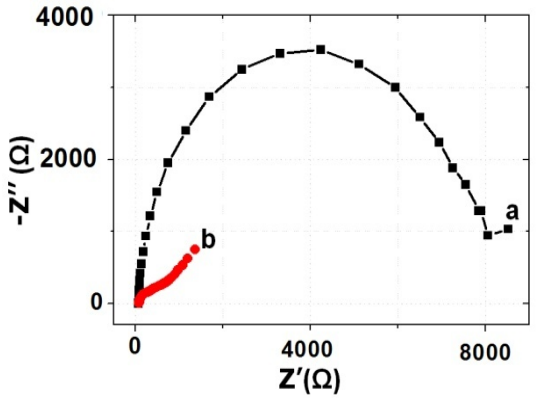


Fig. 3 Nyquist plot of electrochemical impedance spectra from bare SPCE (a) and SPCE-Chi-CeO_2 (b).

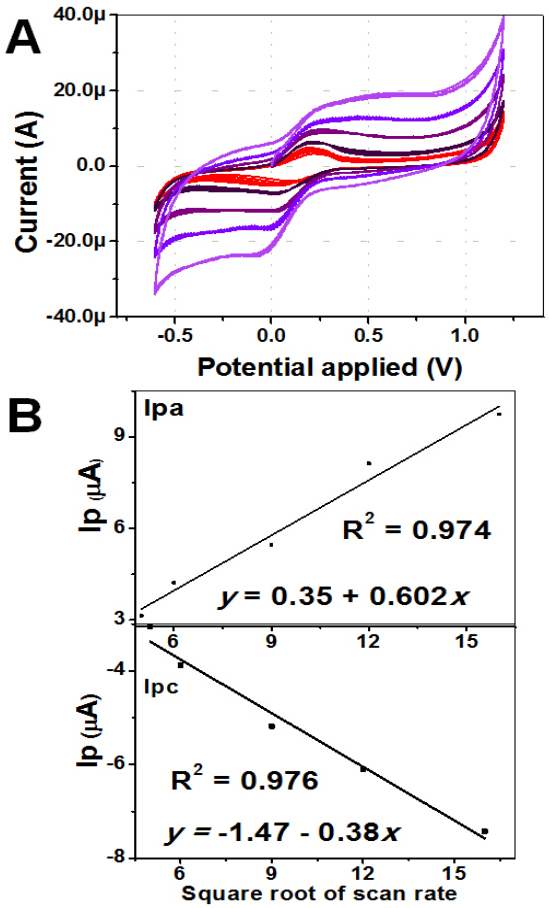


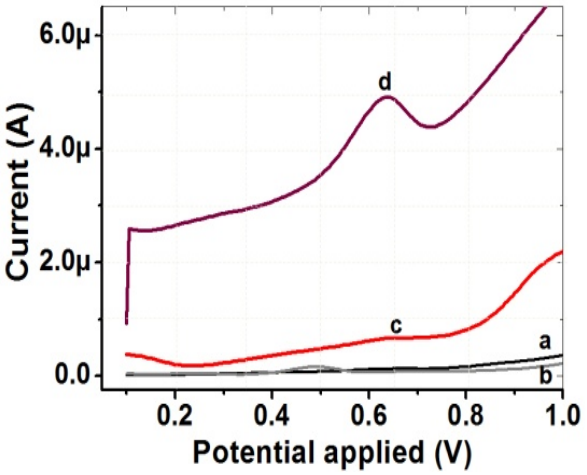
Fig. 4 Cyclic voltammograms of SPCE-Chi-CeO_2 in response to an electrochemical probe $K_3Fe(CN)_6^{3-/4-}$accounted by curves from inner to outer corresponding to 25, 36, 81, 144 and 255 mV/s scan rates, respectively (A), with their coordinate linear relationship of anodic and cathodic peak current vs. square root of scan rate (B).

The fast electron transfer property of SPCE-Chi-CeO_2 could also be affirmed by CV testing, as shown in Fig. 4. It can be seen that typical current peak curves for the oxidation and reduction versus potential applied were arisen in all performing scan rate of 25, 36, 81, 144 and 255mV/s. The current peaks slide apart a bit with increasing scan rate as a characteristic of quasi-reversible reaction of the electrode against the $K_3Fe(CN)_6^{3-/4-}$ redox probe (Fig. 4A). The peak potential (*Ep*) of oxidative and reductive currents was about 0.2 and 0.04 V, respectively. The peak separation was about 0.09 V with a potential window from -0.2 to 1.2 V, while the anodic and cathodic peak currents responses were linearly proportional to the square root of the investigated scan rate with the regression coefficient about 0.9 (Fig. 4B). Thus the

modified SPCE-Chi-CeO_2 is certified satisfactory efficient for further use in electrochemical sensing according to its approved linear diffusion control mechanism and fast electron transfer ability [8].

3.3 Electrochemical oxidation of melatonin

An electrochemical oxidation of melatonin on the screen printed carbon electrode before and after surface modification with cerium oxide-chitosan nanocomposite was investigated using DPV analysis. Fig. 5 shows the DPV of bare SPCE in the absence (curve a) and the presence (curve b) of melatonin at 1 µg/ml in 0.1 M PBS pH 7.0 electrolyte as a sample at the scan rate of 50 mV/s, meanwhile the DPV of SPCE-Chi-CeO_2 in this absence and presence of melatonin is shown up by curve c and d, respectively. The current curves in the presence of melatonin produce one oxidation peak at*E*p around 0.5-0.6 V with peak height (*I*p) about 0.1µA from bare SPCE and 1.6 µA from SPCE-Chi-CeO_2, whereas almost none peak current is exhibited in the absence of melatonin. Obviously higher response and well defined melatonin oxidation peak has been obtained in the case of SPCE-Chi-CeO_2 indicating an efficiently



improvement by this working surface modification.

Fig. 5 Differential pulse voltammetric responses of bare SPCE and modified SPCE-Chi-CeO_2: (a) bare SPCE at 0 µg/ml melatonin, (b) bare SPCE at 1.0 µg/ml melatonin, (c) SPCE-Chi-CeO_2at 0 µg/ml melatonin, and (d) SPCE-Chi-CeO_2 at 1.0 µg/ml melatonin, all in PBS pH 7.0 based electrolyte.

3.4 Quantitative determination of melatonin

Quantitative melatonin determination performance of the SPCE-Chi-CeO_2 was further examined by DPV with reference to its high current sensitivity and a small noise contribution from previous experiment. The peak current tended to increase with the increase of melatonin concentration. A linear relationship between peak current and melatonin concentration was obtained in the range from 1 to 10µg/ml in measurement by bare SPCE with the regression equation of

$y = 2.067 + 0.44x$

where$y = I$p in µA, x = concentration in µg/ml, and **a** correlation coefficient (R^2) = 0.99 (Fig. 6A). Much lower concentration range down to 0.1 to 1.0 µg/ml could be measured by the SPCE-Chi-CeO_2 with the regression equation of

$y = 1.484x - 0.074$, and$R^2 = 0.96$ (Fig. 6B).

The limit of detection, calculated by three times the standard deviation of the linear regression ($S_{y/x}$) divided by the slope value (b), was 1.07µg/ml from the use of SPCE and 0.18µg/ml from SPCE-Chi-CeO_2. Much higher sensitivity was achieved with SPCE-Chi-CeO_2 in consideration of the slope value and LOD results due to a better affinity of Ceo_2-chitosan nanocomposite for melatonin molecules that enhanced an electrochemical response performance. The relative standard deviation (RSD) of each calibration experiments was less than 10% indicating acceptable reproducibility. Although it seems that this LOD is not as low as some other recent reports such as the LOD of 0.02µM (0.005 µg/ml) by CV measurement with multi-walled carbon nanotube (MWCNT) modified glassy carbon electrode [9], the LOD of 0.1µM (0.025 µg/ml) by square wave voltammetry (SWV) with boron-doped diamond microelectrode [10], and the LOD of 0.004µM (0.001µg/ml) by DPV oncarbon ionic liquid electrode modified with MWCNT and cobalt hydroxide nanoparticles [11], but the ease of preparation and a facile operation for ready use with considerable lower cost in comparable to satisfactory detection range for the onsite practical use has made the SPCE-Chi-CeO_2 enduring attraction.

3.5 Selectivity in terms of interference study

To examine the relative reactivity and selectivity of the fabricated SPCE-Chi-CeO_2 toward melatonin detection, the electrochemical DPV were performed with 0.1 M PBS (pH 7.0) supporting electrolyte containing a fixed amount of 10µg/ml melatonin in the presence of other two closely structural related biological indoles as tryptophan and serotonin, adding at similar concentration. Usually, direct measurement of melatonin in presence of indole compounds especially of tryptophan would be impossible due to the proximity of the potentials of their oxidation peaks [12]. However, successful discrimination among them has been testified with SPCE-Chi-CeO_2 as displayed in Fig.7 by the DPV responsein

a mixture of melatonin with tryptophan (curve a), and the mixture of melatonin, tryptophan, and serotonin (curve b).

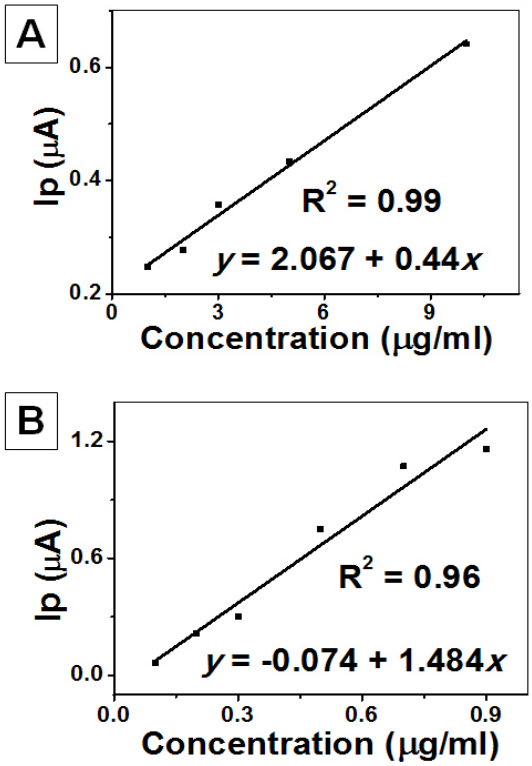


Fig. 6 Calibration plots for melatonin determination from DPV responses with bare SPCE (A) and with SPCE-Chi-CeO_2 (B).

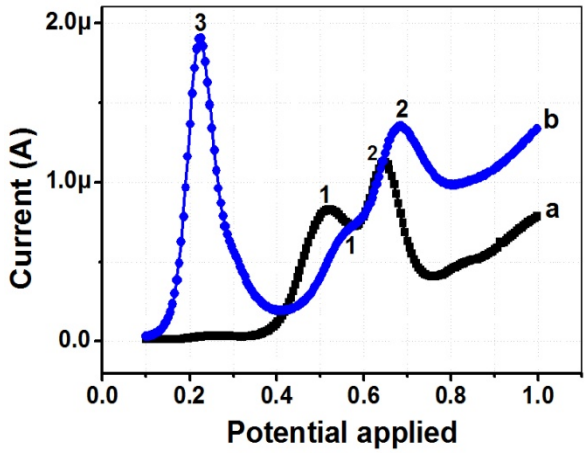


Fig. 7 Differential pulse voltammograms of SPCE-Chi-CeO_2 in 0.1 M PBS (pH 7.0) supporting electrolytecontaining melatonin in the presence of tryptophan (a), and in the presence of tryptophan and serotonin (b) at 10 μg/ml of each compound.

A well separate oxidation peak of melatonin from that of tryptophan can be observed as shown in curve a, in which the typical oxidation peak of melatonin occurs at *E*p around 0.53 V (peak 1), whereas the tryptophan's peak has placed at the next*E*p of 0.65 V (peak 2). Slightly shift in *E*p is apparent in the mixture of melatonin and its two precursors, tryptophan and serotonin which probably due to the competition in the diffusion rate to the electrode surface. The current peak concerning to melatonin exhibits at *E*p of 0.56 V and tryptophan originated peak appears at *E*p around 0.69 V, meanwhile the oxidation peak corresponding to serotonin at *E*p of 0.23 V is evident (peak 3). This phenomenon suggests that melatonin could still be selectively determined in the presence of both indolic precursors. In addition, the modified SPCE-Chi-CeO_2 has allowed feasibility foreffective simultaneous detection of these compounds.

4. CONCLUSION

An effective working electrode SPCE-Chi-CeO_2 was fabricated based on a combination of SPCE and cerium oxide-chitosan nanocomposite for rapid detection of a circadian rhythm regulation hormone, melatonin. Signified characteristics of the fabricated electrodes were morphologically and electrochemically manifested through SEM, EDS, EIS and CV analyses. A prominent improvement in electrochemical response signal was obtained in comparison to the original bare SPCE. Satisfactory working performances in melatonin inspection with rapid response and considerably low detection limit, reproducibility as well as good selectivity have also been verified by quantitative DPV and interference testing with its structural related tryptophan and serotonin. Melatonin detection by this electrode via DPV has shown simple and easy to operate, providing great potential application for practical use. Moreover, this system has allowed simultaneous speciation analysis of these three related indole compounds within the same time, as well as a tool extensible for elaboration of an easy and direct electrochemical determination of melatonin and other hormones in pharmaceutical preparations.

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