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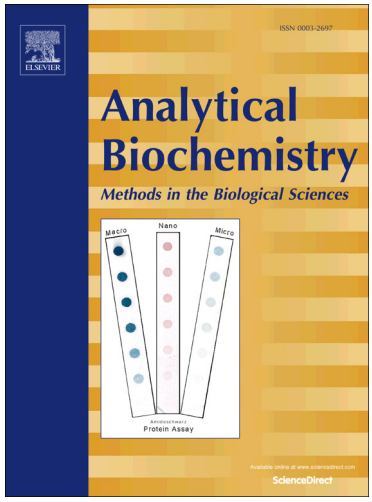

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Development of tyrosinase biosensor based on quantum dots/chitosan nanocomposite for detection of phenolic compounds

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Abbreviations used: QDs, quantum dots; Chit, Chitosan; PBS, Phosphate buffer solution; SEM, scanning electron microscope; Tyr, tyrosinase; GCE, glassy carbon electrode; EIS, Electrochemical impedance spectroscopy; R_{ct}, electron-transfer resistance; E_a, apparent activation energy; K_M^{app}, Michaelis-Menten constant; RSD, relative standard deviation.

Abstract:

A sensitive and simple amperometric biosensor for phenols was developed based on the immobilization of tyrosinase into CdS quantum dots/chitosan nanocomposite matrix. The nanocomposite film with porous nanostructure, excellent hydrophilicity and biocompatibility resulted in high enzyme loading, and the tyrosinase (Tyr) immobilized in this novel matrix retained its activity to a large extent. The CdS quantum dots/chitosan nanocomposite film was characterized by scanning electron microscope and electrochemical impedance spectroscopy, and the parameters of the various experimental variables for the biosensor were optimized. Under the optimum conditions, the designed biosensor displayed a wide linear response to catechol over a concentration range of 1.0×10^{-9} to 2.0×10^{-5} M with a high sensitivity of 561 ± 9.7 mA M^{-1} and a low detection limit down to 0.3 nM at the signal to noise ratio of 3. The CdS quantum dots/chitosan nanocomposites could provide a novel matrix for enzyme immobilization to promote the development of biosensing and biocatalysis.

Keyworeds: Quantum dots; Nanocomposite; Phenolic compounds; Amperometric detection

Introduction

Phenolic compounds including phenol and simple substituted phenol derivatives are widely used in various production processes such as pesticides, plasticizers, petrochemical products and wood preservatives [1]. Many of these compounds as a large group of pollutants are present in the medical, water and food matrices, and most of them are extremely harmful to human health through oral, dermal, or respiratory tracks [2-4]. Therefore, it is important to develop simple method for rapid determination of trace phenolic compounds due to their toxicity and persistency in the environment.

Some methods including spectrophotometry [5], liquid chromatography [6,7] and gas chromatography [8] have been employed to detect the phenolic compounds in water samples. However, some of these techniques are time consuming, complex to perform and expensive, which limit their application for rapid and real-time detection. Electrochemical biosensors have been considered to be a promising method for in situ and rapid determination of phenols due to the high sensitivity, simplicity, and easy to miniaturization [9-12]. One of crucial factors during these biosensors fabrication is the effective immobilization of enzyme in a suitable matrix on electrode surface. Therefore, it is critical to search for materials as effective enzyme immobilization matrixes to design phenols biosensor with a high analytical performance.

Since the discovery of carbon nanotubes, nanomaterials such as grapheme [13,14], magnetic nanobead [15], gold nanoparticle [16,17], polymer [18,19] and quantum dots [20,21] have received wide research interest in bioassays due to their electronic, optical, and mechanical properties. Among these nanomaterials, quantum dots (QDs) have been extensively used for optical and electrochemical biosensing because of their unique advantages such as nanoscale size similar to proteins, much higher specific surface, and versatility in surface modification, which are favorable for the immobilization of

biomolecules [22-26]. Chitosan is a nontoxic natural biopolymer with a large amount of positive charges. It has been widely used for protein immobilization to develop biosensors due to its special properties including excellent film forming ability and good biocompatibility [27-29].

In the present work, we developed a nanocomposite film based on CdS QDs and Chitosan (Chit) as a host matrix for tyrosinase immobilization to design an amperometric biosensor for sensitive and rapid detection of phenols. This nanocomposite film displayed several advantages for enzyme immobilization such as good hydrophilicity and biocompatibility, high specific surface, and good film-forming capability. The designed biosensor based on the nanocomposite matrix exhibited excellent analytical performances for the detection of phenolic compounds such as high sensitivity, wide linear range, subnanomolar detection limit and good repeatability.

Experimental

Reagents and chemicals

Tyrosinase (EC 1.14.18.1, 1000 U mg^{-1}) from mushroom, and mercaptopropionic acid were purchased from Sigma-Aldrich. Catechol, *p*-cresol, phenol, *m*-cresol, *p*-chlorophenol and chitosan (Chit, 90% deacetylation) were obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Other reagents were of analytical grade and were used without further purification. All aqueous solutions were prepared with twice-distilled water. Phosphate buffer solution (PBS) was 0.1 M Na_2HPO_4 and NaH_2PO_4, and its pH was adjusted with H_3PO_4 or NaOH solutions. Phenolic solutions in 0.1 M PBS were prepared daily.

Apparatus and characterization

Electrochemical studies were performed with CHI660d electrochemistry workstation

(Shanghai CH Instrument Co., China). The electrochemical measurements were carried out with a three-electrode system. A saturated calomel electrode (SCE) and a Pt foil electrode were used as the reference electrode and the counter electrode, respectively, and modified glassy carbon electrode was the working electrode. Micrographs were obtained with a JSM-7001F scanning electron microscope (SEM). The static contact angles were measured with a contact angle meter (Rame-Hart-100) using droplets of deionized water at 25°C. Electrochemical impedance spectroscopic was studied in 0.1 M PBS containing 10 mM $Fe(CN)_6^{3-/4-}$ and 0.1 M KCl with a frequency range of 0.01 Hz–10 kHz.

Preparation of the CdS QDs/Chit nanocomposite and enzyme electrodes

The water-soluble CdS QDs were prepared using mercaptopropionic acid as stabilizing agent according to a reported method [30]. Chit solution (0.1% w/w) was prepared by dissolving Chit flakes in 1.0 % acetic acid and was then diluted into 0.1% solution by adding twice-distilled water. Then 40 μM of CdS QDs was mixed with Chit solution at a 1:1 (volume ratio) and sonicated for 10 min. Subsequently, 4.0 mg of tyrosinase (Tyr) was dissolved in 1.0 mL of CdS QDs/Chit mixture solution. The glassy carbon electrode (GCE) was polished carefully using 0.3 and 0.05 μm alumina slurry followed by rinsing thoroughly with distilled water. After successive sonication in 1:1 nitric acid, acetone and distilled water, the electrode was allowed to dry at room temperature. Then, 15 μL of the mixed solution (CdS QDs/Chit/Tyr) was spread on the surface of the GCE, and then dried at 4°C in a refrigerator.

Protocols and statistical data treatment

In this experiment, all electrochemical measurements were carried out in a thermostated cell at 25 °C, containing 0.1 M PBS. The current response for the biosensor was obtained by injecting of catechol into the PBS at -0.2 V. The average results of all tables were obtained

with three replicates based on three different biosensors prepared by the same steps. The date of all the figures was acquired using three different electrodes constructed by the same procedure and tested with the same reactants and the same sample. The error bars in the whole manuscript were standard deviation.

Results and discussion

Characterization of Chit, QDs/Chit nanocomposite films

The surface morphologies of Chit and QDs/Chit composite films were characterized by SEM imaging. As shown in Fig. 1A, the morphology of the pure Chit film exhibited an excellent film-forming character with irregular wrinkle. Compared to pure Chit film, the morphology of QDs/Chit nanocomposite film displayed a porous structure (Fig. 1B). This obvious difference could be attributed to the interaction between the negatively charged CdS QDs and positively charged Chit molecules to produce aggregation. This nanocomposite film might integrate the merits of its precursors and would be suitable for enzyme immobilization with advantages such as good biocompatibility and adhesion, and controlled porosity.

The biocompatibility of a host matrix for loading of enzymes and preserving their bioactivity is positively related to its hydrophilicity which can be studied by the contact angle measurement of the material. As shown in inset of Fig. 1, the contact angle of CdS QDs/Chit nanocomposite film was measured to be 30°, which was smaller than that of pure Chit film (57°). This demonstrated that the QDs/Chit nanocomposite film possessed better hydrophilicity than that of pure Chit film due to the abundant carboxylic acid groups on QDs surface. Thus, the high activity of the entrapped enzymes could be kept on this nanocomposite film, which is highly advantageous to the preparation of biosensors.

Electrochemical impedance spectroscopy (EIS) is an effective technique to study the interface properties of surface-modified electrodes. Fig. 2 displays a modified Randle's

equivalent circuit and the Nyquist plots of the impedance spectroscopy of the different electrodes. The semicircle diameter equals the electron-transfer resistance (R_{ct}), which represents the electron transfer kinetics of redox probe at the electrode interface. The R_{ct} values of bare GCE, Chit/GCE, QDs/Chit/GCE, and QDs/Chit/Tyr/GCE were obtained as 280, 1837, 1507 and 2450 Ω (curves a-d) respectively. The R_{ct} value of QDs/Chit/GCE (curve c) was smaller than that of Chit/GCE (curve b), demonstrating that the nanocomposite film allowed greater permeation for the redox probe of $Fe(CN)_6^{3-/4-}$ than the pure Chit film. An obvious increase in the resistance was observed when Tyr was immobilized in the QDs/Chit film (curve d), which was caused by the hindrance of the macromolecular structure of Tyr to the electron transfer.

Optimization of measurement variables

The experimental variables, which can affect the amperometric detection of phenols, include the pH of PBS, applied potential, and temperature of the detection system. The effect of pH on the QDs/Chit/Tyr electrode response to 4 μM catechol over the pH range from 5.0 to 8.5 was studied. The current firstly increased as the pH increased from 5.0 to 6.5, followed by decreasing in the pH range of 6.5 to 8.5. The maximum current signal of the designed biosensor was obtained at pH 6.5. This pH effect was consistent with the wide optimal pH range of 5.0 to 8.0 reported for the free enzyme. Therefore, PBS at pH 6.5 was selected as the electrolyte in subsequent experiments.

Fig. 3 displays the influence of applied potential on the amperometric response of the biosensor to 4 μM catechol. In the range of 0.1 to -0.25 V, the maximum response was obtained at -0.2 V, while more negative potentials led to a decrease of the current response. This decrease could be attributed to an increase in the direct consumption of oxygen on the electrode surface, which leaded to oxygen depletion in the vicinity of the Tyr immobilized on

the electrode surface, and finally to decrease the enzymatic rate. Therefore, -0.2 V was selected as the optimal applied potential in subsequent experiments.

The effect of temperature on the biosensor response to 4 μM catechol was investigated in the range of 3 to 45°C. The current increased as the temperature changed from 3 to 30°C, afterwards the current response decreased as the temperature further increased. Maximum current response was obtained at about 30°C. At higher temperatures, the current response decreased due to the denaturation of the Tyr embeded on electrode. In the range of 3 to 25°C a plot of ln *i versus* T^{-1} showed a straight line. According to the Arrhenius equation, the apparent activation energy (E_a) of the enzyme catalytic reaction, calculated from the slope of the straight line, was 41 kJ mol^{-1}. This value was similar to that of 38.8 kJ mol^{-1} for the Tyr/polyaniline-ionic liquid-carbon nanofiber electrode [31]. For a simple experimental procedure and long lifetime of the designed biosensor, all other measurements were performed at 25°C.

Amperometric detection of phenolic compounds

Fig. 4 shows a typical current-time plot for the amperometric biosensor by successively adding of catechol into the PBS with pH 6.5 at -0.2 V. The current signal immediately achieved 95% of steady-state current within 5 s after the addition of catechol, which indicated a fast mass transfer of the substrate across the nanocomposite film and a quick electron exchange between Tyr and its substrates, demonstrating the advantages of the QDs/Chit coating as matrix for the fabrication of biosensor.

The inset in Fig. 4 shows the calibration curve of the QDs/Chit/Tyr electrode response to catechol. A wide linear response to catechol was obtained ranging from 1.0×10^{-9} to 2.0×10^{-5} M with a correlation coefficient of 0.997. A low detection limit of 0.3 nM catechol was determined at a signal-to-noise ratio of 3. The sensitivity of the proposed biosensor response

to catechol was 561 ± 9.7 mA M^{-1}, which was much higher than that of biosensor based on a hybrid material of chitosan and layered double hydroxides as matix (194 mA M^{-1}) [32], graphene oxide conjugated with Tyr assembled gold nanoparticles (160 mA M^{-1}) [33], Tyr entrapped in chitosan-carbon-coated nickel nanocomposite film (514 mA M^{-1}) [10], Tyr embedding in single-wall carbon nanotubes and polyaniline nanocomposites (244 mA M^{-1}) [34], and Tyr immobilized in bismuth nanoparticles (44.1 mA M^{-1}) [35]. The high sensitivity and low detection limit of the QDs/Chit/Tyr electrode may be attributed to the advantages of QDs/Chit nanocomposite, including good hydrophilicity and biocompatibility.

The designed biosensor also exhibited sensitive amperometric responses to the phenolic compounds such as *p*-cresol, phenol, *m*-cresol and *p*-Chlorophenol. The detection characteristics of the biosensor for the different phenolic compounds, including linear range, correlation coefficient, sensitivity, and detection limit, were listed in Table 1. The sensitivity in the linear calibration region for the substrates was in the following order: catechol > *p*-cresol > phenol > *m*-cresol > *p*-Chlorophenol, which was in accord with the previous reports [3]. The apparent Michaelis-Menten constant, K_M^{app}, refelcts information on the enzyme-substrate kinetics for the enzyme electrode: the smaller of the K_M^{app} value, the greater of the affinity to substrate. The K_M^{app} values obtained according to the Lineweaver–Burk equation were 5.21, 4.75, 5.13, 3.84 and 2.91 μM for catechol, *p*-cresol, phenol, *m*-cresol and *p*-Chlorophenol, respectively. These values were significantly lower than the reported for free enzyme (300 μM) [36]. The low K_M^{app} value indicated that the Tyr entrapped in the matrix of QDs/Chit composite had better affinity to phenolic compounds.

Repeatability, reproducibility and stability of the QDs/Chit/Tyr electrode

The repeatability of the QDs/Chit/Tyr electrode was examined by consecutive measurements of its response to 4 μM catechol. As shown in Fig. 5, the current response of

the proposed biosensor with a relative standard deviation (RSD) of 4.5% was observed in 50 successive measurements, which proved good repeatability of the biosensor. The reproducibility of the designed biosensor was analyzed using eight different electrodes constructed by the same procedure for measurement of 4 μM catechol. The result revealed that the RSD value for all eight electrodes was 5.1%. The long-term stability of the Tyr/CNS electrode stored at 4°C was evaluated periodically using the same PBS containing 1 μM catechol (inset in Fig. 5). The current response of the QDs/Chit/Tyr electrode was unchanged during two weeks of storage, and it still retained about 80% of its original response after 50 days of storage. The good stability of the QDs/Chit/Tyr electrode was attributed to the good hydrophilicity and biocompatibility of the QDs/Chit composite matrix.

Interference and real water samples analysis of QDs/Chit/Tyr electrode

The influence of some possible interfering substances on the proposed biosensor was investigated by using a 0.2 μM catechol standard solution in the presence of different interferents. The interferents with concentration 100 times of that of catechol, including caffeine, aniline, glucose, PO_4^{3-}, Mg^{2+} and Ca^{2+}, were added separately into 0.2 μM catechol standard solution. The proposed QDs/Chit/Tyr electrode exhibited no significant interference in the presence of the above species, indicating good selectivity to phenolic compounds detection.

To demonstrate the potential application of this biosensor in analysis of real samples, the designed biosensor was applied in determination of catechol spiked in real water samples with the standard addition method. The two water samples obtained from the local river (Jinshan Lake) and tap water were spiked with three different concentrations of catechol. As shown in Table 2, the recoveries ranged from 94.1% to 105.3% for the spiked samples, which indicated the satisfactory accuracy of the developed biosensor and confirmed the application

potential of the designed biosensor to measure phenols in real samples.

Conclusion

In this paper, a simple and facile enzyme electrode based on the immobilization of Tyr in QDs/Chit nanocomposite matrix has been developed for the sensitive determination of phenolic compounds. The QDs/Chit nanocomposite film combining of the advantages of two components can provide a good reasonable permeability and biocompatible microenvironment for the immobilized enzyme, and keep the entrapped enzyme with high affinity to substrates. The proposed biosensor displays good analytical performances such as high sensitivity, wide detection range, good repeatability and rapid response. The QDs/Chit nanocomposite material provides an effective matrix for enzymes and biomolecules loading, which has a great potential for multiple applications in biosensors and other electrically active devices.

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Table 1 The response characteristics of the QDs/Chit/Tyr modified electrode to various phenolic compounds (average values were obtained with three replicates based on three biosensors).

Phenolic compound	Linear range/M	R^2	Sensitivity (mA M^{-1})	Detection limit (nM)	K_M^{app}(μM)
Catechol	1.0×10^{-9} – 2.0×10^{-5}	0.997	561 ± 9.7	0.3	5.21
p-Cresol	1.0×10^{-9} – 2.0×10^{-5}	0.998	514 ± 8.5	0.5	4.75
Phenol	6.5×10^{-9} – 3.0×10^{-5}	0.997	462 ± 6.1	1.0	5.13
m-Cresol	1.0×10^{-8} – 1.5×10^{-5}	0.998	388 ± 8.4	2.5	3.84
p-Chlorophenol	1.5×10^{-8} – 8.0×10^{-6}	0.996	235 ± 7.9	4.0	2.91

Table 2 Determination of spiked catechol in different real water samples using the proposed biosensor (n = 3).

Water sample	Catechol (M)		Recovery	RSD,
	Added	Found	(%)	(%)
Jinshan lake	9.0×10^{-7}	8.7×10^{-7}	96.7	4.9
	4.0×10^{-6}	4.2×10^{-6}	105.3	3.7
	6.5×10^{-6}	6.7×10^{-6}	103.5	4.2
Tap water	9.0×10^{-7}	8.5×10^{-7}	94.1	4.5
	4.0×10^{-6}	3.9×10^{-6}	97.8	3.6
	6.5×10^{-6}	6.6×10^{-6}	101.4	2.8

Figure captions

Fig. 1. SEM images and contact angles (inset) of (A) Chit film and (B) CdS QDs/Chit nanocomposite film.

Fig. 2. Nyquist plots of EIS for (a) bare GCE, (b) Chit/GCE, (c) QDs/Chit/GCE, and (d) QDs/Chit/Tyr/GCE in 0.1 M PBS containing 0.1 M KCl and 10 mM $[Fe(CN)6]^{3-/4-}$ for a frequency range of 0.01 Hz–10 kHz. Inset: the equivalent circuit applied to fit the impedance spectra.

Fig. 3. Influence of applied potential on the biosensor response to 4 μM catechol in 0.1 M pH 6.5 PBS.

Fig. 4. Typical current-time response curve of the QDs/Chit/Tyr electrode upon successive additions of catechol in 0.1 M PBS with pH 6.5, at -0.2 V. Inset: calibration curve of the biosensor for catechol detection.

Fig. 5. Repeatability of QDs/Chit/Tyr electrode response to 4 μM catechol under the optimal condtions. Inset: storage stability for the designed biosensor.

Figure 1

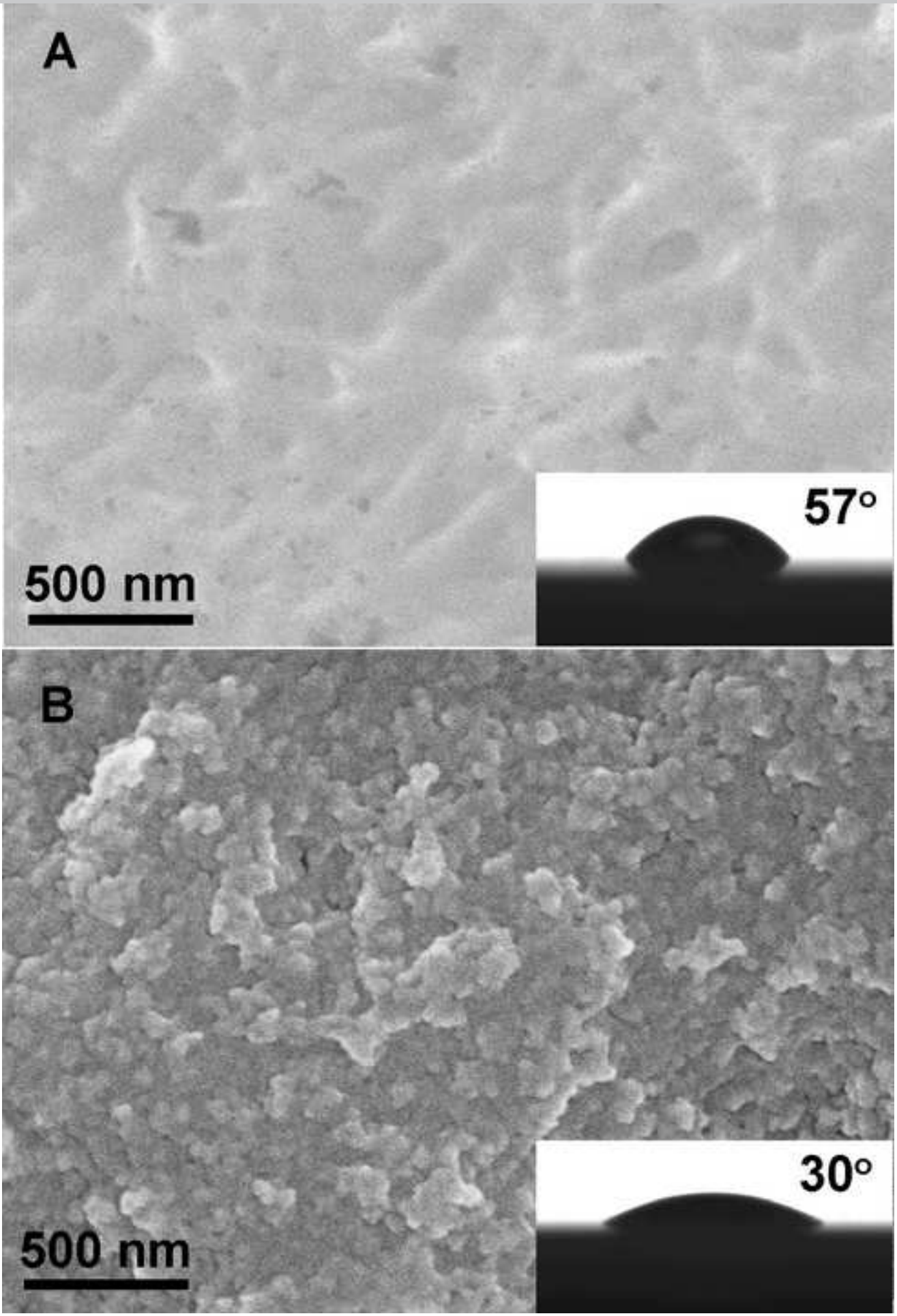

Figure 2

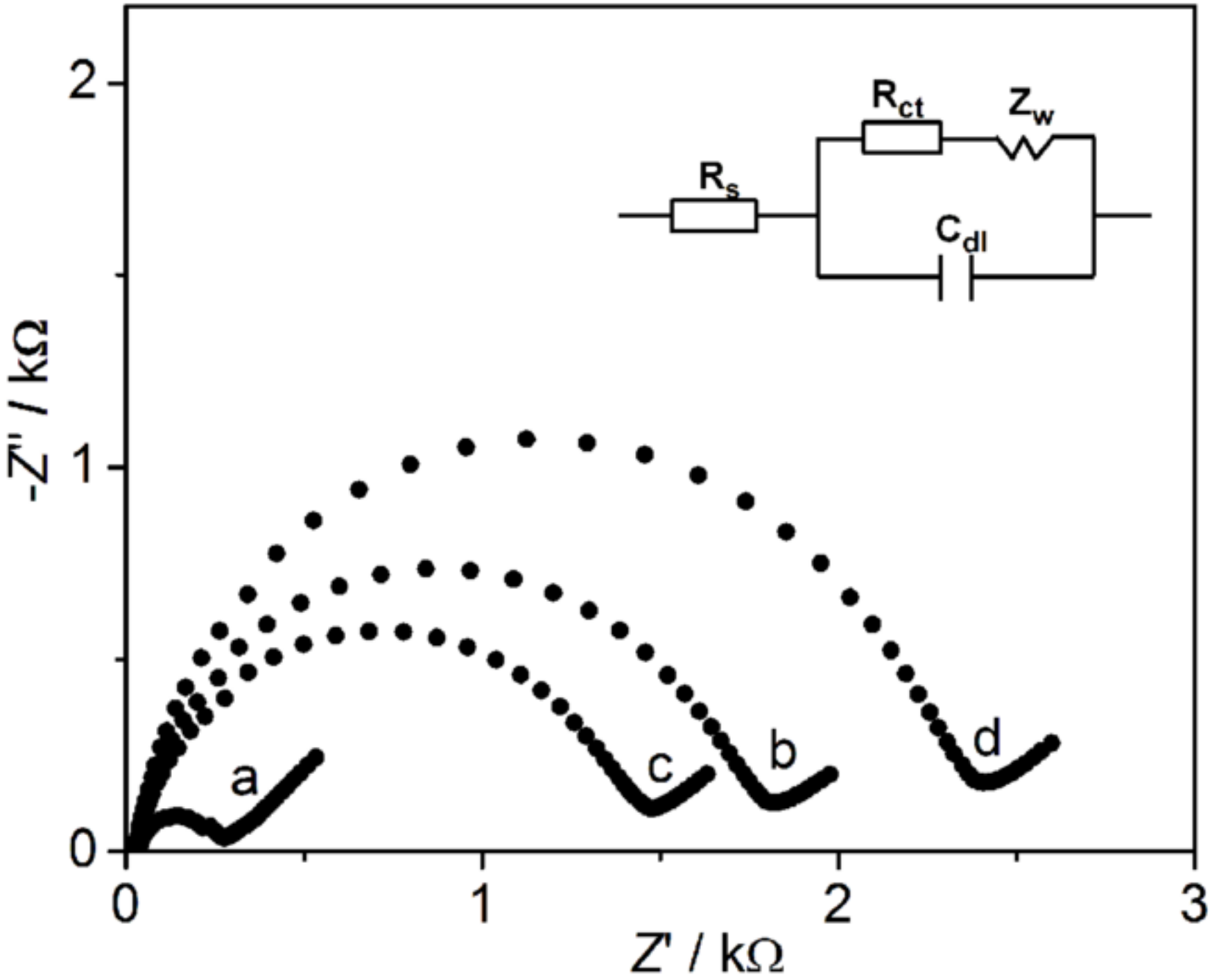

Figure 3

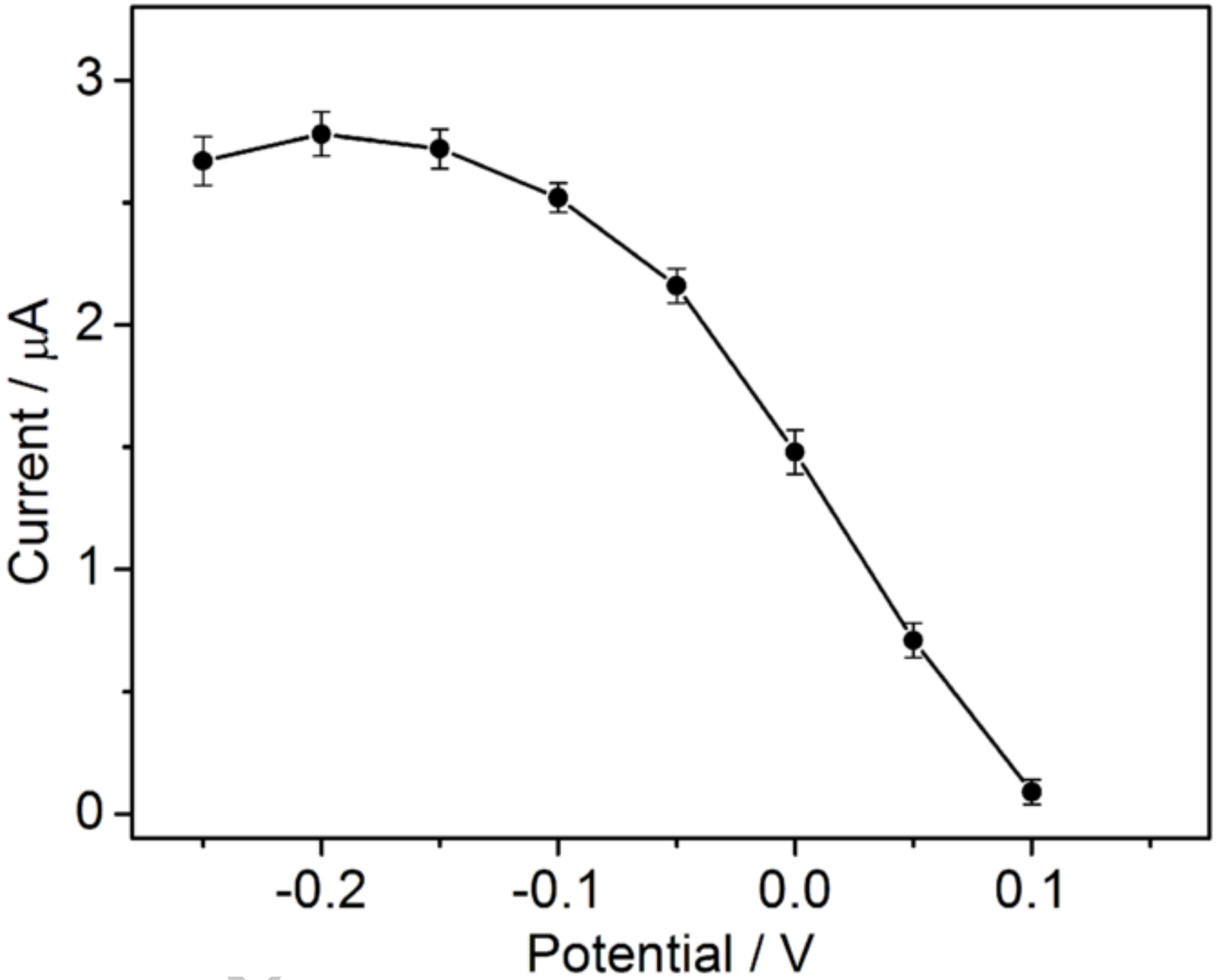

Figure 4

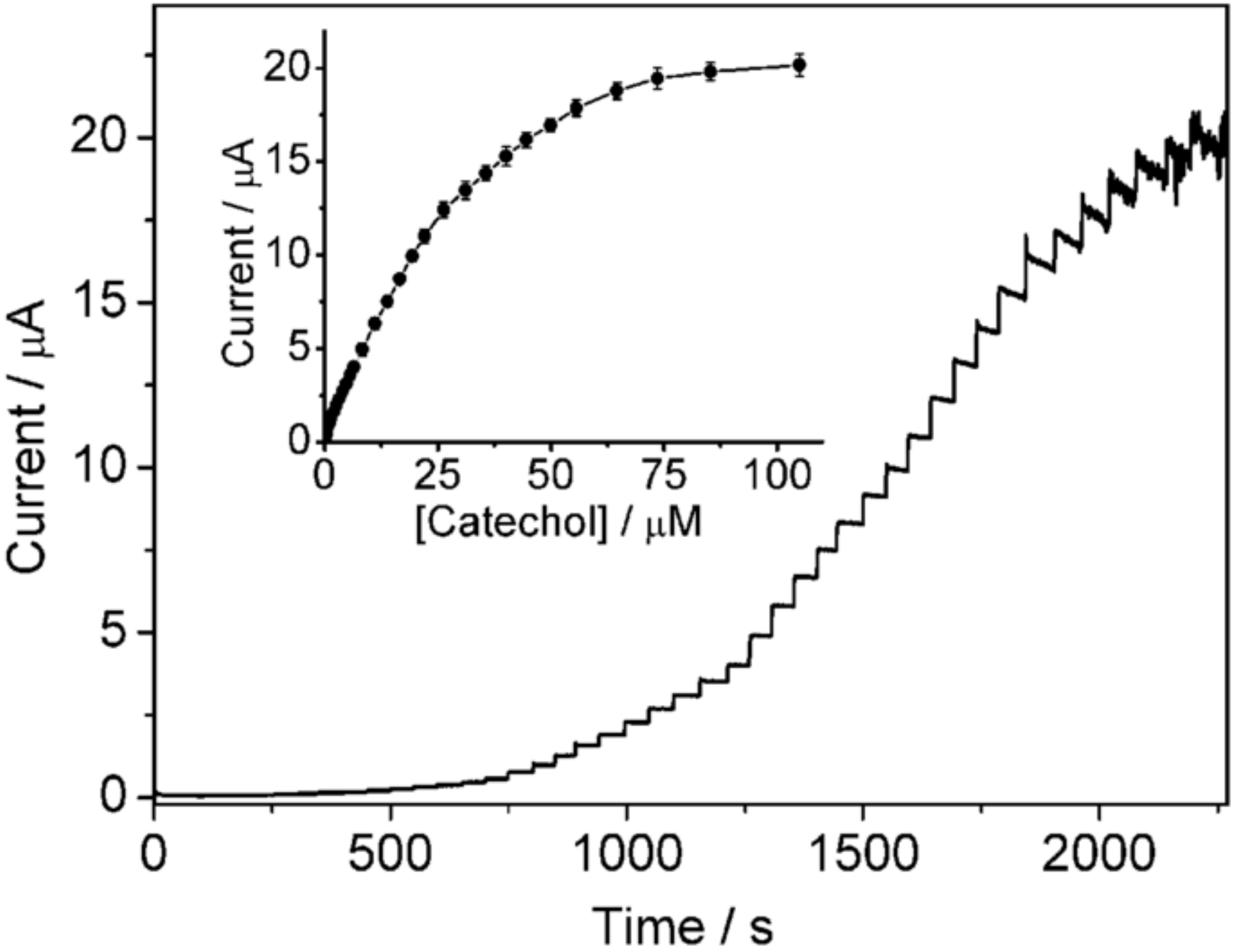

Figure 5