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CHARACTERIZATION OF CELLULOSE NANOCRYSTAL-GOLD NANOPARTICLES/CHITOSAN MODIFIED SCREEN-PRINTED CARBON ELECTRODE AND ITS APPLICATION IN THE FABRICATION OF ELECTROCHEMICAL BIOSENSOR FOR TETRACYCLINE DETECTION

(Percirian Elektrod Skrin Bercetak Karbon Terubahsuai Nanokristal Selulosa-Partikel Nano Emas/Kitosan dan Aplikasinya dalam Fabrikasi Penderia Bio Elektrokimia untuk Pengesanan Tetrasiklin)

Nurul Shahirah Hasim[1], Nor Azah Yusof[1,2], Ruzniza Mohd Zawawi[1], Noordiana Nordin[3*]

[1]Department of Chemistry,
Faculty of Science,
Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[2]Functional Nanotechnology Devices Laboratory,
Institute of Nanoscience and Nanotechnology,
Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[3]Laboratory of Food Safety and Food Integrity,
Institute of Tropical Agriculture and Food Security,
Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia.

[*]Corresponding author: noordiana@upm.edu.my

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Abstract

Tetracycline is one of the antibiotics used therapeutically and as a growth promoter in animals. Tetracycline residues in food products of animal origin have raised concerns among consumers. Due to its adverse effects on human health, it is critical to develop a reliable analytical method for routine monitoring of tetracycline in foods. Biosensors are among the rapid analytical devices that are reliable because of their simple detection methodology, low cost, sensitivity and specificity. For an effective signal transformation of tetracycline residues, it is critical to completely attach the transducer to the biological component, so that a feasible biosensor can be constructed. In this study, the screen-printed carbon electrode (SPCE) was modified with cellulose nanocrystal–gold nanoparticles/chitosan composite (CNC–AuNPs/chitosan). The synthesized CNC–AuNPs composite was characterized using UV–Vis spectroscopy, X-ray diffraction, Fourier-transform infrared spectroscopy and high-resolution transmission electron microscopy. In addition, the electrochemical behavior of the modified SPCE was investigated by cyclic voltammetry, differential pulse voltammetry (DPV) and electrochemical impedance spectroscopy under optimized conditions. DPV showed a linear calibration within the range of 0.01 to1000 µM concentration of tetracycline with the detection limit of 0.07 µM. The developed biosensor also resulted in different peak currents measured after storage for 14 days at room temperature (53.07%) and under 4°C (89.28%). Therefore, with acceptable sensitivity and selectivity, the fabricated biosensor can be suggested as a potential detection method for tetracycline residues.

Keywords: Cellulose nanocrystal, gold nanoparticles, screen-printed carbon electrode, electrochemical biosensor, tetracycline

Abstrak

Tetrasiklin adalah salah satu antibiotik yang digunakan secara terapeutik dan sebagai penyokong pertumbuhan pada haiwan. Sisa tetrasiklin dalam produk makanan yang berasal dari haiwan telah menimbulkan kebimbangan di kalangan pengguna. Oleh kerana kesan buruknya terhadap kesihatan manusia, adalah sangat penting untuk membangunkan kaedah analisis yang boleh dipercayai untuk pemantauan rutin tetrasiklin dalam makanan. Penderia bio adalah antara peranti analisis pantas yang boleh dipercayai kerana kaedah pengesanannya yang mudah, menjimatkan kos, kepekaan dan kekhususannya. Untuk transformasi isyarat yang berkesan dari sisa tetrasiklin, adalah sangat penting untuk melampirkan transduser sepenuhnya ke komponen biologi, supaya penderia bio yang boleh dilaksanakan dapat dibina. Dalam kajian ini, penderia bio dengan pengubahsuaian elektrod skrin bercetak karbon (SPCE) telah melibatkan nanokristal selulosa-partikel nano emas/kitosan (CNC-AuNPs/kitosan). Komposit CNC-AuNPs yang disintesis telah dicirikan dengan menggunakan spektroskopi penglihatan-UV, pembelauan sinaran-X, spektroskopi inframerah transformasi Fourier dan mikroskopi penghantaran elektron. Di samping itu, tingkah laku elektrokimia SPCE yang diubahsuai telah disiasat oleh voltammetri berkitar, voltammetri pembeza denyut (DPV) dan spektrometer electrokimia impedans dibawah keadaan yang optima. DPV menunjukkan kalibrasi linear dalam julat kepekatan tetrasiklin 0.01 hingga 1000 μM dengan had pengesanan 0.07 μM. Penderia bio yang telah dibangunkan juga telah menghasilkan perbezaan dalam arus puncak yang diukur setelah disimpan selama 14 hari pada suhu bilik (53.07%) dan di bawah suhu 4°C (89.28%). Oleh itu, dengan sensitiviti dan selektiviti yang telah diperoleh, penderia bio yang dibangunkan disyorkan berpotensi sebagai kaedah pengesanan sisa tetrasiklin.

Kata kunci: nanokristal **s**elulosa, partikel nano emas, elektrod karbon tercetak, penderia bio elektrokimia, tetrasiklin

Introduction

Antibiotic residues in animal-derived foods, owing mostly to antibiotic abuse in animal husbandry, have become a global concern and a significant health risk for humans and animals [1]. Since the 1940s, tetracycline has played a significant role in veterinary medicine and feed additives because of its broad-spectrum antibacterial properties and economic benefits. In livestock agriculture, tetracycline is frequently used to stimulate growth through increased food intake, weight gain and herd health. This approach may result in the presence of trace levels of these medications in animal-derived products such as milk and eggs, posing substantial health hazards to humans. The presence of tetracycline residues in foods may result in the transmission of antibiotic-resistant pathogenic microorganisms via the food chain. Another effect identified is its potential to trigger allergic reactions in hypersensitive persons [2].

According to Food and Drug Administration summary reports, sales and distribution of antimicrobials authorized for use in food-producing animals totaled roughly 14.8 million kg in 2012, with tetracycline accounting for 40% of total sales [3]. This data demonstrates that tetracycline is widely employed in animal food production, placing it at the top of the list of antibacterial drugs. To assure food safety and protect consumers from exposure to these contaminants, national and international regulatory organizations have established a tolerance level for tetracycline. Maximum residue levels (MRLs) are the maximum permissible concentrations of food residues and/or hazardous metabolites in food and feed products [4]. Food and Agricultural Organization of United Nations/World Health Organization have determined MRLs for tetracycline to be 200 μg kg^{-1} in chicken muscle and 400 μg kg^{-1} in egg samples [5].

Due to the possible detrimental effects of tetracycline in food, it is vital to develop accurate analytical methods that are speedy, precise, cost-effective, time-saving and environmentally friendly for routine tetracycline monitoring in food. Biosensor technology is being implemented in the field of analytical chemistry and is rapidly gaining popularity among researchers. Biosensors are small, portable devices that enable the detection of analytes by converting the chemical reaction to a signal utilizing biological recognition elements adsorbed on the physicochemical transducer [6]. This signal may generate qualitative or quantitative information. Biosensors have become one of the most often used rapid analytical methods, owing to the advantages of being quick, simple and sensitive. As a

result, the integration of biosensors currently has not only enhanced research on food safety involving chemical and microbial contamination but also improved the preservation of food freshness through packaging methods [7].

One of the fundamental principles of the electrochemical biosensor is the correlation between the biological component and the transducer that may affect the specificity of detection. Several previous reports have utilized nanomaterials as a significant advancement in these areas [8–11]. In this study, we propose a novel cellulose nanocrystal–gold nanoparticles/chitosan (CNC–AuNPs/chitosan) as the modifier of the screen-printed carbon electrode (SPCE) to amplify the current signal measured. Using the modification, an electrochemical biosensor was fabricated to determine tetracycline residues. The differential pulse voltammetry (DPV) technique was used in the presence of a redox indicator during detection.

Materials and Methods

Reagents and apparatus

All the chemical reagents used in this study were of analytical grade and used without further purification unless stated otherwise. Dry CNC with 0.94 weight percentage of sulphur (Batch# 2014-FPL-CNC-065) was purchased from the University of Maine (USA). Chitosan, tetracycline hydrochloride and streptomycin sulphate were procured from Nacalai Tesque (Japan). Gold (III) chloride trihydrate ($HAuCl_4.3H_2O$), bovine serum albumin (BSA), trizma hydrochloride (Tris–HCl) and glutaraldehyde (GA) were obtained from Sigma-Aldrich (USA). Potassium chloride (KCl) was obtained from Bendosen Chemical (Malaysia). Potassium ferricyanide ($K_3Fe(CN)_6$) and phosphate buffer saline (PBS) were purchased from R&M Chemicals (Malaysia). Potassium ferrocyanide ($K_4Fe(CN)_6$) was purchased from Millipore (Canada). Synthetic oligonucleotides for amine-terminated ssDNA (tetracycline aptamer, TET-aptamer) were purchased (as lyophilized powder) from Apical Scientific (Malaysia) with the following sequence: 5'-NH_2–CCCC CGGC AGGC CACG GCTT GGGT TGGT CCCA CTGC GCGT-3' (anti-tetracycline). Electrochemical measurements were performed using an Autolab III Eco-Chemie (Netherland) voltammetric analyser with NOVA 1.11 software and an impedance potentiometric module FRA 32M (10 μHz to 32 MHz) (Netherland) with NOVA 2.4 software. The SPCE used for the analysis, which comprises a three-electrode system: a counter electrode, a carbon working electrode and an Ag/AgCl reference electrode, was purchased from Metrohm DropSens (Spain). The characterization was performed using UV–vis spectroscopy (Perkin Elmer), X-ray diffraction (XRD) (Philips), high-resolution transmission electron microscopy (HR-TEM) (JEOL) and Fourier-transform infrared spectroscopy (FTIR) (Shimadzu).

Synthesis and characterization of CNC-AuNPs

CNC–AuNPs were synthesized by mixing 1% sodium citrate solution with 0.2 mM $HAuCl_4$ solution and 0.1% CNC suspension. The volume of the mixture was made up of deionized water and heated at 80°C for an hour. The color changes were observed, and the final solution was then cooled to room temperature and kept in a fridge. The CNC–AuNPs were characterized using UV–vis spectroscopy, XRD, HR-TEM and FTIR.

Fabrication and characterization of modified SPCE

The SPCE was modified based on the layer-by-layer method. First, CNC–AuNPs were sonicated for 5 min. Then, 4 μL of CNC–AuNPs was drop cast on the SPCE (SPCE/CNC–AuNPs) and dried at ambient temperature. Chitosan solution was prepared by adding 0.5 g chitosan to 50 mL acetic acid (2%) and stirred for several hours until completely dissolved. Then, 4 μL chitosan solution was drop cast as the second layer (SPCE/CNC–AuNPs/chitosan) and air-dried. The bare and modified SPCE was characterized after each deposition layer using cyclic voltammetry (CV) at the potential range of –0.4 to 0.7 V for 20 cycles with a scan rate of 0.1 V/s in 1 mM $K_3Fe(CN)_6$ and 50 mM KCl. The electrochemical impedance spectroscopy was conducted at the frequency range of 0.01 to 10 000 Hz with an amplitude of 0.3 V in 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$ and 0.1 mM KCl.

Development of electrochemical biosensor

The modified SPCE was placed in 1% GA and washed. The GA acts as the linker between the DNA probe and

the modified SPCE. Various TET-aptamer concentrations ranging from 0.01 to 1000 nM were drop cast on the SPCE/CNC–AuNPs/chitosan and kept at room temperature at various incubation times ranging from 15 to 90 min. Optimum concentration and incubation time of the TET-aptamer were selected for further analysis. Tris–HCL buffer was used to wash the unbound aptamer on the SPCE/CNC–AuNPs/chitosan/TET-aptamer prior to measurement by DPV. Several concentrations of tetracycline ranging from 10 nM to 1 mM were drop cast on the SPCE/CNC–AuNPs/chitosan/TET-aptamer and incubated for an hour. Tris–HCL buffer was used to wash the unbound tetracycline. Prior to electrochemical measurement, the SPCE/CNC–AuNPs/chitosan/TET-aptamer was immersed in BSA to avoid non-specific binding. DPV was performed at the potential range of –0.6 to 0.7 V and a scan rate of 0.01 V/s in 1 mM $K_3Fe(CN)_6$ and 50 mM KCl.

Results and Discussion

Characterization of CNC-AuNPs

UV–vis spectroscopy was used to confirm the presence of AuNPs in CNC suspension. The CNC–AuNPs nanocomposite, as shown in Figure 1A, exhibited a distinct absorption peak around 525 nm, which can be traced back to the surface plasmon resonance band of AuNPs. The broad peak area is expected from the synthesis of AuNPs using a citrate-reduction method [12]. The cellulose nanocrystal structure of the synthesized CNC–AuNPs was determined using XRD. Figure 1B depicts the XRD patterns of CNC and CNC–AuNPs. The CNC showed a prominent peak at 2 theta at 15.2°, 22.6° and 34.5°, which were derived from the typical reflection planes (110), (200) and (040) of cellulose, respectively [13]. With the addition of those peaks, CNC–AuNPs displayed four new peaks in the vicinity of 39.1°, 44.2°, 64.9° and 77.8°, which were indexed to the (111), (200), (220) and (311) planes of gold lattice, respectively [14]. Consequently, the XRD pattern indicated that the metallic production of AuNPs did not change the crystallographic property of CNC. The FTIR technique was used to determine the chemical composition or structure of the sample. In most cases, the CNC is represented by a broad peak of O–H spanning between 3100 and 3700 cm^{-1} due to the hydrogen connection between the hydroxyl group and the carboxyl group. The presence of a hydrogen bond in the structure aids in the nucleation process that occurs during the synthesis of AuNPs [15]. As illustrated in Figure 1C, the CNC–AuNPs displayed peaks at roughly 2900 cm^{-1} and 1635 cm^{-1}, which were attributed to the C–H stretching and the C=C stretching of the cellulose structure, respectively, in the spectrum.

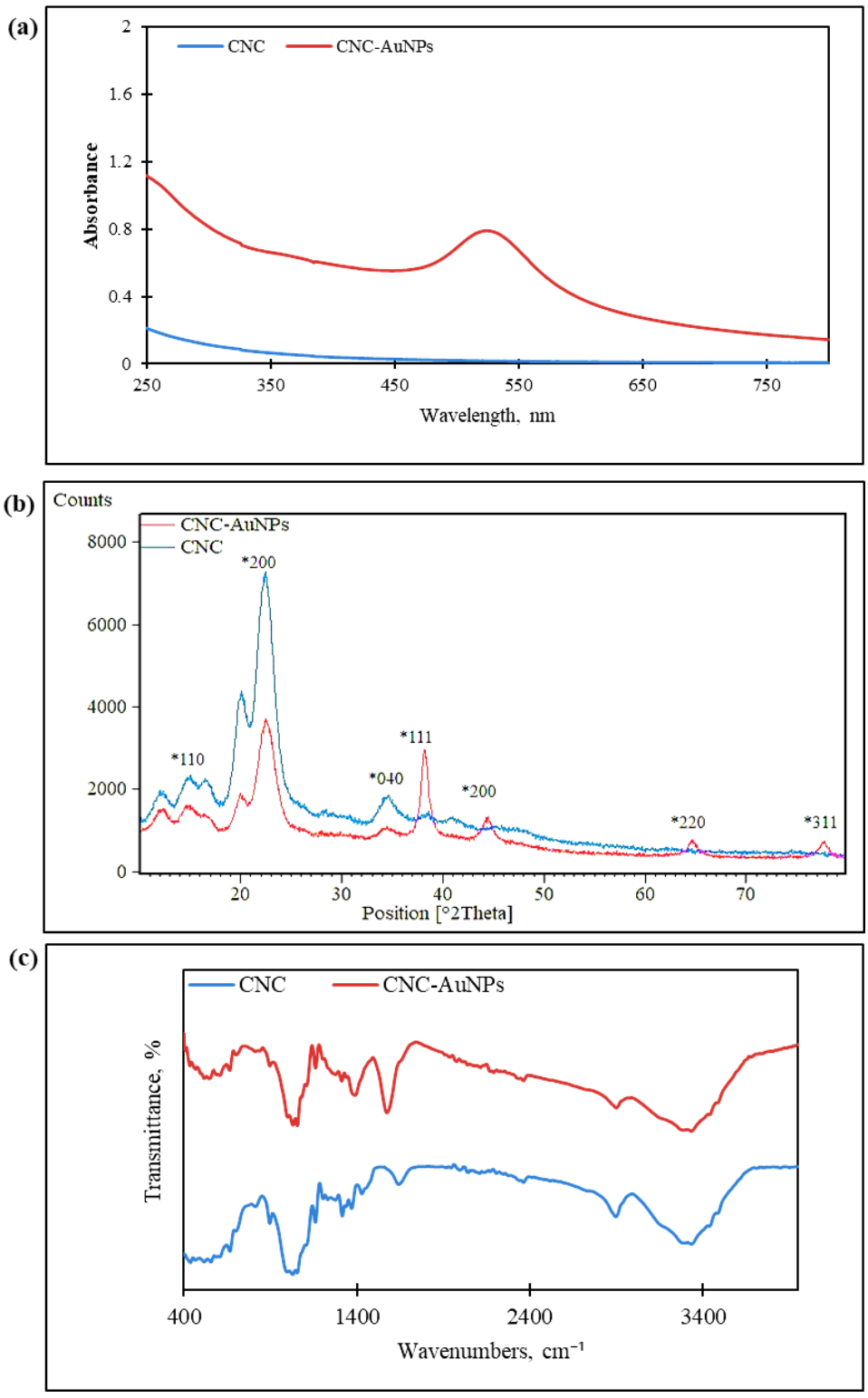

Figure 1. (a) UV-vis spectra, (b) XRD pattern and (c) FTIR spectra of CNC and CNC-AuNPs

The TEM images clearly revealed the production of the gold particles. In Figure 2a, CNC's TEM image revealed rod-like particles of cellulose crystalline with a diameter of less than 10 nm. The distribution showed that most of the cellulose crystals are about 6 to 7 nm. Meanwhile, Figure 2b reveals that there is a small populated zone of AuNPs with a diameter of around 20 nm. The agglomeration and the inconsistency in the particle size of the AuNPs may be due to the inefficient thermal process during the synthesis [16]. In addition, the CNC–AuNPs elemental spectra were obtained using the TEM–EDX. The EDX analysis revealed an abundance of gold particles with a weight percentage of 84.2, indicating that the production of AuNPs in the presence of CNC has occurred.

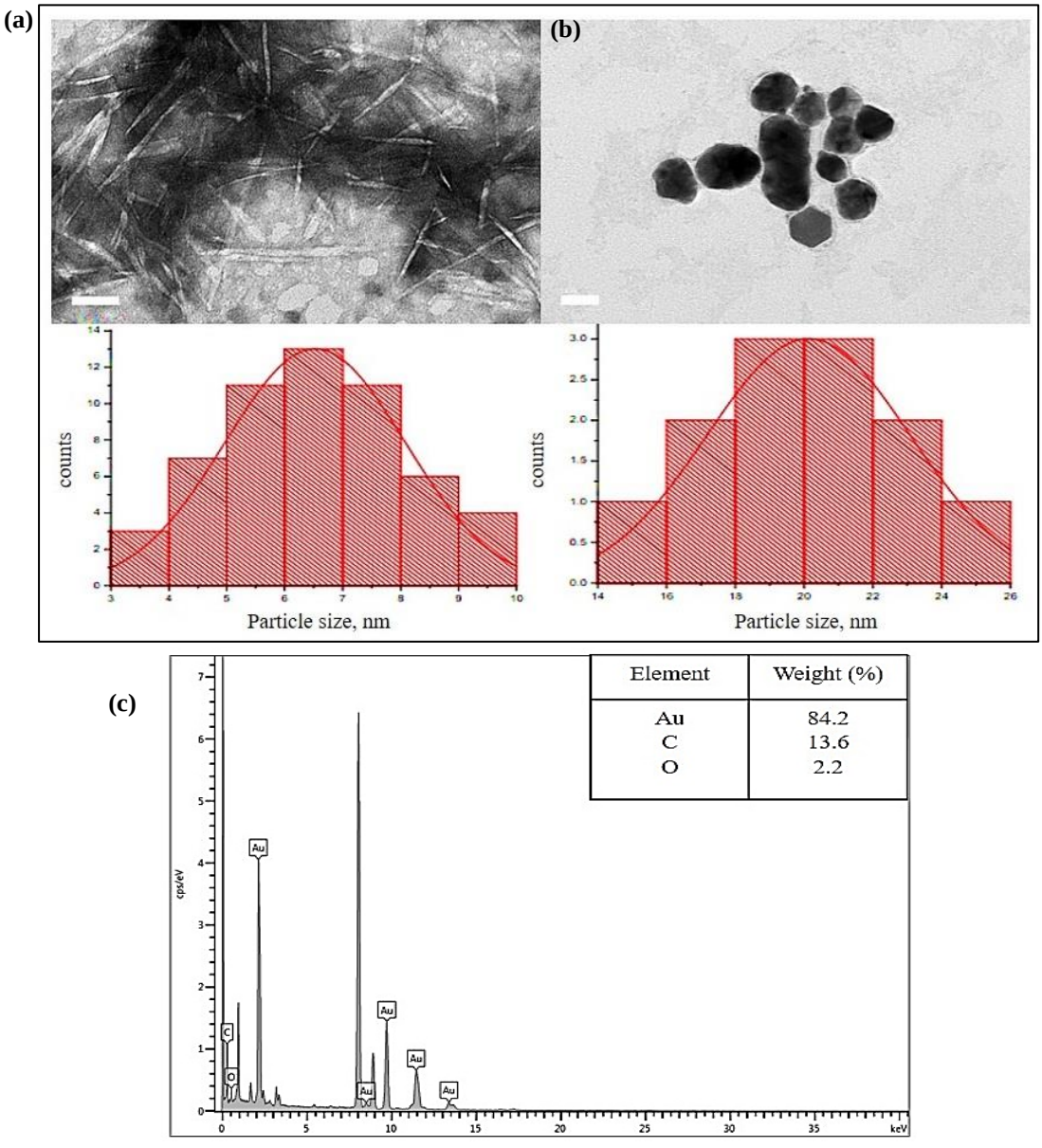

Figure 2. (a) TEM image of CNC, (b) TEM image CNC-AuNPs (50k magnification) and (c) TEM-EDX analysis of CNC-AuNPs (inset; elemental analysis)

Electrochemical properties of the modified electrode

For the modification of the SPCE, chitosan solution was drop cast on the CNC–AuNPs surface. CV was performed to investigate the bare SPCE and CNC–AuNPs/chitosan-modified SPCE, and the signals are shown in Figure 3a. The CNC–AuNPs/chitosan-modified SPCE demonstrated the highest anodic peak, measuring around 74.29 µA when compared with the CNC–AuNPs-modified SPCE. The increment of about sevenfold in the peak current may be due to the chitosan layer that acts as a hydrophobic barrier [17], allowing the CNC–AuNPs to remain intact on the surface of SPCE, thus allowing the amplification of the signal. By calculating the relative standard deviation (RSD), the repeatability and reproducibility of the modified SPCE were determined. The percent RSD for repeatability was 5.1% when five consecutive readings of the modified electrode were taken. Using the same approach on five individual electrodes resulted in a 6.4% RSD for reproducibility. As for the Nyquist plot from Figure 3b, it is proved that the measurement complimented the CV measurements as the resistance decreased for the modified electrode. The R_{ct} values recorded for bare and modified electrodes were 394.08 and 116.27 Ω, respectively. Therefore, it is believed that the modified electrode can improve conductivity while also providing reliable and reproducible measurements for the biosensor.

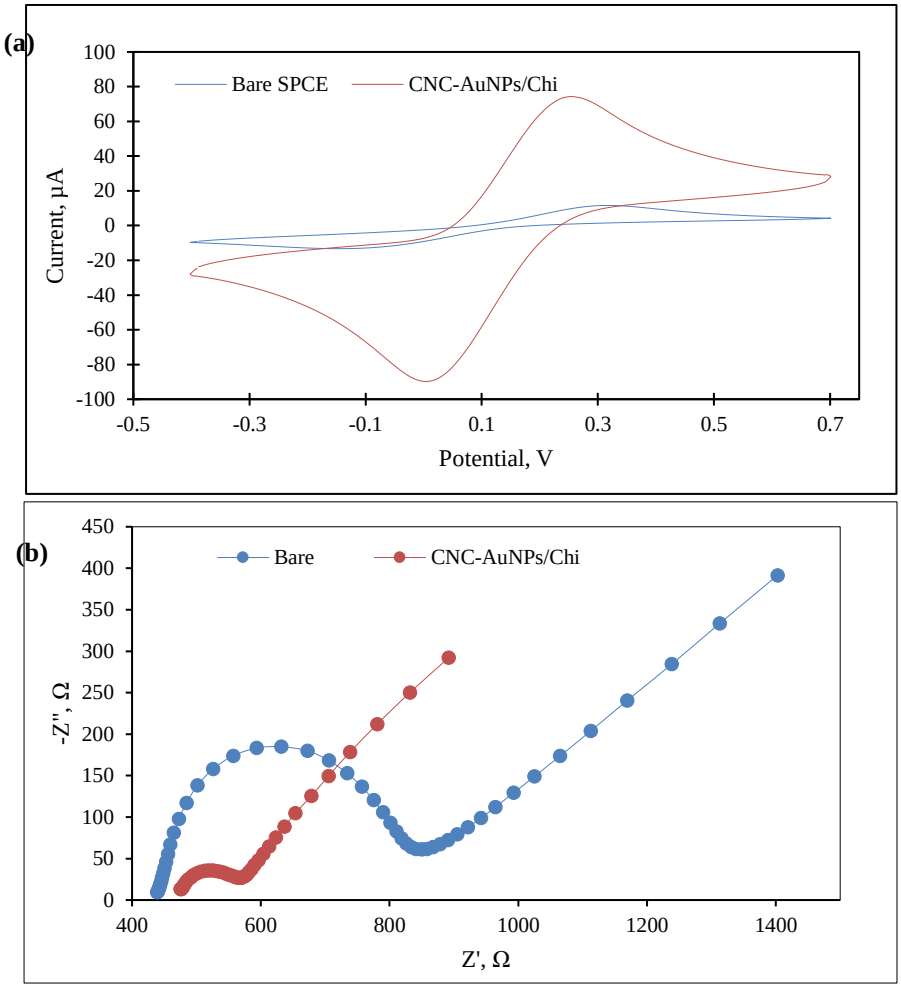

Figure 3. (a) Cyclic voltammogram and (b) Nyquist plot of bare SPCE and CNC-AuNPs/chitosan-modified SPCE

Optimization of the TET-aptamer

Several variables, including incubation duration and immobilized TET-aptamer concentration, were investigated to obtain the highest possible output from the biosensor. These parameters must be optimized because they have an impact on the binding of the TET-aptamer with the tetracycline as well as the peak current of DPV. To find the optimal concentration of the TET-aptamer, the CNC–AuNPs/chitosan-modified SPCE was pipetted with various concentrations (0.01 to 1000 nM) of the TET-aptamer for 30 min. Based on Figure 4a, the peak current increased as the concentration of the TET-aptamer increased from 0.01 to 1 nM. Nevertheless, the peak current began to decrease as the concentration increased from 10 to 1000 nM. This could be due to the too close proximity on the surface of the CNC–AuNPs/chitosan-modified SPCE that causes electrostatic repulsion. Hence, the optimized concentration of the TET-aptamer was found to be 1 nM. In addition, Figure 4b shows the effect of the incubation time of the TET-aptamer on the DPV peak current. The TET-aptamer (1 nM) was dropped on the CNC–AuNPs/chitosan-modified SPCE for 15 to 90 min. The findings showed that the peak current reached a maximum at 75 min yet decreased at 90 min, which might be due to the instability of the TET-aptamer after being left for a longer period on the electrode surface. Thus, the optimum incubation time of the TET-aptamer was selected at 75 min.

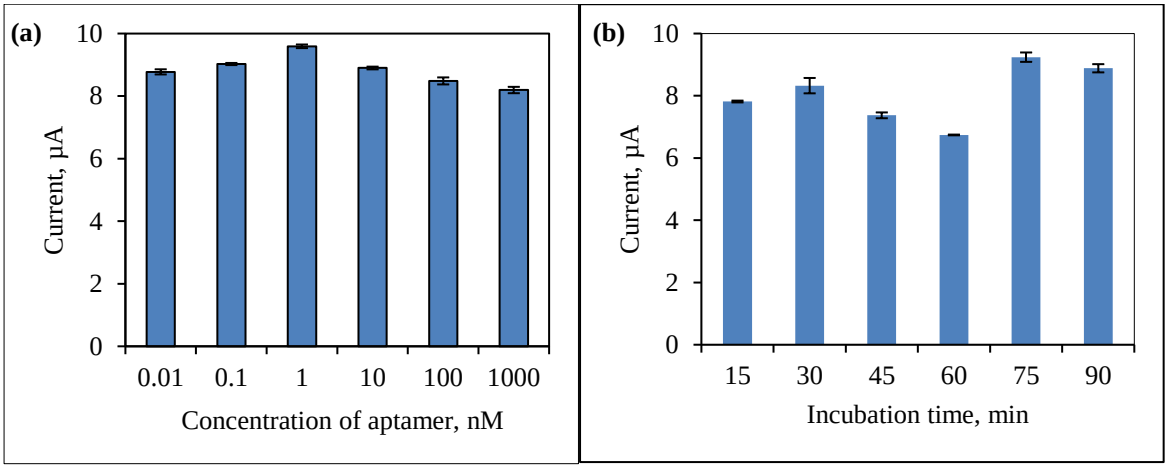

Figure 4. The effect of different **(A)** incubation times and **(B)** concentrations of the TET-aptamer at potential of -0.6 to 0.7 V

Detection of tetracycline

Under the optimal experimental conditions, the DPV response was recorded for the tetracycline detection. To validate the constructed biosensor, tetracycline was incubated on the immobilized TET-aptamer surface at multiple concentrations ranging from 10 nM to 1 mM. It can be observed in Figure 5a that the continuous drop of the DPV peak current was associated with the binding of the tetracycline to the TET-aptamer. These findings also can be compared with those of Zhao et al. [18], Benvidi et al. [19], and Tang et al. [20] who reported a decrease in DPV signals with the presence of tetracycline. In addition, the linear calibration was plotted between the log of different concentrations of tetracycline and the relative change in peak current ($\Delta I/I_0$) (Figure 5b). The biosensor demonstrated a linear regression, which was stated as $y = 0.70x + 4.65$ with a correlation coefficient of $R^2 = 0.9707$. From the linear slope, the limit of detection (LOD) was obtained at 0.07 µM of tetracycline concentration, which was calculated using the $3\sigma/s$ formula, where σ is the standard deviation of the blank and s is the slope of the calibration curve. The reproducibility of the developed biosensor was also evaluated from five individual electrodes and the resulted percent RSD was 4.1%. This proved the consistency of the signal measurements, which relate to the reliability of the fabricated biosensor.

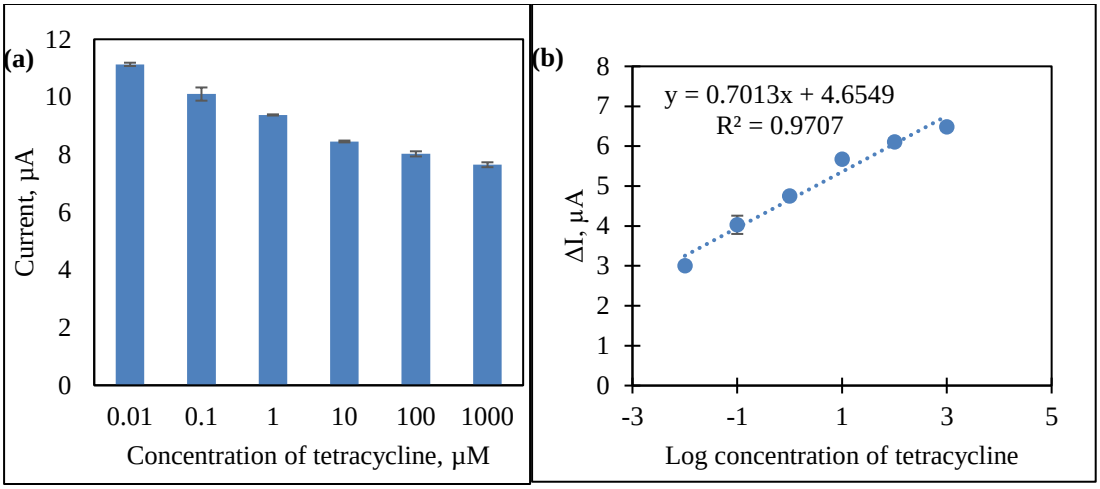

Figure 5. (a) DPV of the biosensor taken at different concentrations of tetracycline and (b) the calibration curve of the biosensor; LOD: 0.07 µM

The selectivity of the biosensor was investigated using several types of antibiotics to assess the specificity of the TET-aptamer binding to the tetracycline. DPV responses to tetracycline, ampicillin, streptomycin and oxytetracycline are demonstrated in Figure 6. The highest peak current (12.32 µA) was observed for ampicillin, owing to the various binding sites of the TET-aptamer that were used to capture it. Compared with the other antibiotics, tetracycline recorded the lowest peak current (10.10 µA). This demonstrates that the TET-aptamer is capable of distinguishing tetracycline from other distinct antibiotic molecules (ampicillin and streptomycin) as well as the structurally related derivative antibiotics (oxytetracycline). The stability of the developed biosensor was also analyzed using 100 µM tetracycline. The biosensor was stored for 14 days at room temperature and under 4 °C. The relative difference in peak current from the initial response for room temperature and under 4 °C was 53.07% and 89.27%, respectively. This indicates that the biosensors cannot be stored more than 2 weeks before use.

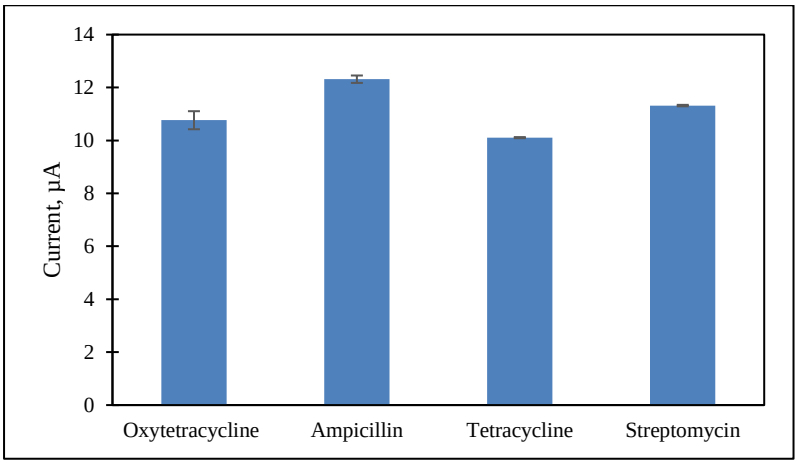

Figure 6. DPV responses of the tetracycline, ampicillin, streptomycin and oxytetracycline

Conclusion

In this study, CNC–AuNPs were synthesized, characterized and employed as the transducer element for the fabrication of a biosensor. After being modified in conjunction with chitosan, the modified SPCE demonstrated a significant current peak increment of sevenfold. With an acceptable percent RSD, the CNC–AuNPs/chitosan-modified SPCE showed a repeatable and reproducible result. With the chosen modified surface, the biosensor was developed for tetracycline detection. DPV recorded a decreasing current with an increasing concentration of tetracycline. From the analysis, it was found that the LOD for the detection was 0.07 μM (RSD = 4.1%). The findings of this study imply that using a combination of approaches to modify disposable SPCEs with CNC–AuNPs and chitosan for prospective use as a biosensor for tetracycline detection may be a potential alternative.

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