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Electrochemical detection of a breast cancer susceptible gene using cDNA immobilized chitosan-*co*-polyaniline electrode

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ABSTRACT

An electrochemical breast cancer biosensor based on a chitosan-*co*-polyaniline (CHIT-*co*-PANI) copolymer coated onto indium–tin-oxide (ITO) was fabricated by immobilizing the complementary DNA (cDNA) probe (42 bases long) associated with the breast cancer susceptible gene BRCA1. Both the CHIT-*co*-PANI/ITO and the cDNA/CHIT-*co*-PANI/ITO electrodes were characterized with Fourier transform infrared (FTIR) spectroscopy, atomic force microscopy (AFM), cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). For the cDNA/CHIT-*co*-PANI/ITO electrode, the amperometric current decreased linearly with an increasing logarithm of molar concentration of the single-stranded target DNA (ssDNA) within the range of 0.05–25 fmol. The bioelectrode exhibited a sensitivity of 2.104 μA/fmol with a response time of 16 s. The cDNA/CHIT-*co*-PANI/ITO electrode had a shelf life of about six months, even when stored at room temperature.

1. Introduction

Breast cancer starts in the breast cells of men and women. Worldwide, breast cancer is the second most common type of cancer and the second-leading cause of cancer death in women. Although considerable advancements have been made in detection and treatment in recent years, the majority of breast cancers are still detected in their last stage of advancement [1,2].

The advent of gene technology provides the hope of discovering novel biological sensors for early breast cancer detection, disease diagnosis, prognostication, and predicting responses to therapy. Abnormalities in the expression of specific genes have been linked to a huge and increasing number of diseases, including cancers. Specific genetic mutations occur in the cancerous breast cells that acquire mutations in the oncogenes and tumor suppressor genes [3].

Most breast cancers have gene mutations; thus, gene expression sensing is a promising technique for early detection. However, the existing nucleic acid detection techniques, including Northern blotting [4], ribonuclease protection assays [5], and reverse transcription-polymerase chain reaction (RT-PCR) [6,7] have limitations, including low sensitivity, poor selectivity and nonlinearity to the target strength [8]. These limitations affect the precision and quality of the biosensor and often provide distorted information on the gene expression for the breast carcinoma.

In order to rapidly detect primary-stage breast carcinoma, there is a significant need for low-cost, sensitive, and selective biosensors. In recent years, electrochemical nucleic acid biosensors have received much attention due to their rapid response, high sensitivity, and inherent selectivity [9–11]. The electrochemical detection technique provides a simple, accurate, and inexpensive platform for molecular detection. This approach is promising for the early detection of breast carcinoma using a breast cancer susceptible gene-1 (BRCA1; 5592 bp) specific cDNA probe (synthesized from mRNA of the cancerous breast tissue using RT-PCR) [12].

The performance of electrochemical biosensors generally depends on the physico-chemical properties of the electrode materials, as well as the sensing element immobilized over the electrode surface [13]. Most of the reported electrochemical biosensors must apply a high positive charge potential on the working electrode, which minimizes the interference effects from the reducing species [14].

Chitosan (CHIT) is a moderately inexpensive and stable electroactive material that allows for mass-production of biosensors. It is one of the most widely used biopolymers for sensor applications due to its nontoxic nature, excellent film forming ability, good mechanical strength, high permeability, and cost-effectiveness [15]. CHIT is a non-conducting biomaterial; however, it has an excellent biocompatible property that favors the immobilization of biomolecules over its surface [16]. The surface immobilization and protection of the biosensing element, i.e., the ssDNA or cDNA probe,

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is one of the key challenges for fabricating a highly sensitive DNA biosensor [17–20]. It was also reported that modifying CHIT with conducting polymers such as polyaniline (PANI) improves its redox properties that influence the electron-transfer kinetics during the course of electrochemical detection [21,22].

PANI is a unique polymer whose variable conductivity can be controlled by the protonation of the imine sites on the main polymer chain [23]. Although pure PANI is not compatible for the genetical specimens, its composites usually provide a sustainable matrix for the biological molecules [24]. It was reported that the sensitivity and selectivity of PANI to genetical substances such as DNA, mRNA, and nucleic acids can be improved through copolymerization with polymers that have a variety of functional groups such as –OH, $–NH_2$, –COOH, and acetyl [25–28]. Thus, CHIT-*co*-PANI has been investigated as a promising sensing electrode for the early detection of breast carcinoma.

During this study, cDNA probe sequences associated with the breast cancer gene BRCA1 was immobilized onto the surface of a CHIT-*co*-PANI/ITO electrode. Using this electrode, the ssDNA was electrochemically detected by a change in current due to DNA hybridization. The advantageous features of this biosensor include a low price, ease of preparation, high sensitivity, and good selectivity.

2. Experimental

Aniline (Aldrich, 99.5%) and CHIT (Aldrich, >85% deacetylated) were used without further purification. All solutions were prepared with 18.2 MΩ deionized water. Indium–tin-oxide (ITO) coated glass (Balzers) sheets with a resistance of 15 Ω/cm were used as substrates for the deposition of CHIT-*co*-PANI copolymer electrodes.

The breast cancer specimens were obtained from a woman undergoing surgery for primary breast cancer. The breast tumor tissues were stored in liquid nitrogen immediately after surgical resection, transported to the laboratory, and then stored at −70 °C until mRNA extractions were performed.

2.1. Preparation of CHIT solution

To prepare the CHIT solution, 1.0 g of CHIT flakes was dissolved into 100 mL of 1.0% acetic acid. The resulting mixture was stirred for 3 h at room temperature until the CHIT flakes were completely dissolved. The CHIT solution was stored in a refrigerator at 4 °C.

2.2. Fabrication of CHIT-co-PANI/ITO electrode

Aniline (150 μL) and 1.0% CHIT solution (85 μL) were mixed with 10 mL of 0.5 M HCl in an electrochemical cell and the mixture was ultrasonically agitated for about 4 h. The CHIT-*co*-PANI was chronoamperometrically synthesized onto an ITO coated glass surface using a three-electrode assembly with the ITO glass as working, platinum as counter, and Ag/AgCl as reference electrodes at a potential of 0.9 V and a duration of 240 s.

2.3. mRNA isolation and cDNA preparation

The mRNA of the breast tumor tissues was extracted by homogenization in Trizol reagent (Invitrogen), and cDNA was prepared using oligo(dT) primer (Boehringer Mannheim) with Superscript™ II reverse transcriptase (Invitrogen) for 50 min at 42 °C [29].

The following sequences of the primers were designed by PCR using SuperScript™ II: target DNA: 5′ AGCTCGCTGAGACTTCCTGGATCC CCCACAGCCGACTACTGA 3′; probe cDNA: 5′ TCGAGCGACTCTGAAGGACCTAGG GGGTGTCGGCTGATGACT 3′; mismatch DNA: 5′ AGCACGCTGAGACTTGCTGGA TCCCCCTCAGCCGACTACAGA 3′.

2.4. Immobilization of cDNA probe onto CHIT-co-PANI/ITO electrode

The CHIT-*co*-PANI/ITO electrode was upturned and 15 μL of cDNA probe (350 nmol) was pipetted over the electrode surface and kept at room temperature for 5 h. The resulting cDNA probed CHIT-*co*-PANI/ITO bioelectrode was thoroughly washed with water and rinsed with a phosphate buffer solution (PBS) of pH 7.0 to rinse off any loosely bound cDNA from the electrode (Fig. 1).

2.5. Hybridization of cDNA onto CHIT-co-PANI/ITO electrode

The hybridization reaction was carried out by pipetting 15 μL of buffer containing 10 mM Tris–HCl, 1.0 mM EDTA, and 0.10 M NaCl on the surface of the electrode hold with a different molar concentration of the ssDNA. The resulting electrode was kept at 42 °C for 50 min, and then washed with PBS of pH 7.0 to remove the unhybridized ssDNA over the CHIT-*co*-PANI/ITO electrode. A similar procedure was adopted for the mismatched ssDNA (Fig. 1).

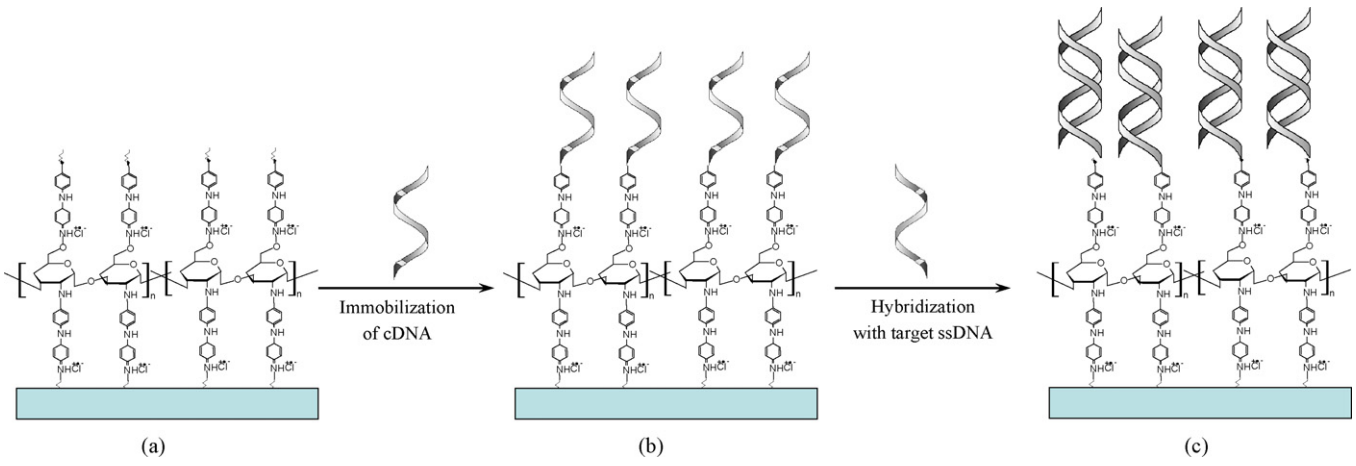


Fig. 1. Schematic presentation of the (a) fabrication of CHIT-*co*-PANI/ITO electrode; (b) immobilization of cDNA (BRCA1) over CHIT-*co*-PANI/ITO electrode; and (c) hybridization of the ssDNA onto cDNA/CHIT-*co*-PANI/ITO electrode.

2.6. Electrochemical detection

The electrochemical detection was performed by cyclic voltammetry (CV) with a three-electrode cell configuration comprising Ag/AgCl as a reference electrode, platinum foil as a counter electrode, and cDNA/CHIT-*co*-PANI/ITO as a working electrode in PBS of pH 7.0 containing 5 mM $Fe(CN)_6^{3-/4-}$ at 30 mV s^{-1} scan rate.

2.7. Characterization

Electrochemical measurements of the electrodes were carried out on a potentiostat/glavanostat (Princeton Applied Research, 273 A) unit with three electrodes in PBS of pH 7.0. The working electrode was one of the following three electrodes fabricated: CHIT-*co*-PANI/ITO; cDNA/CHIT-*co*-PANI/ITO; and target DNA hybridized CHIT-*co*-PANI/ITO. Platinum foil and Ag/AgCl were used as the counter and reference electrodes, respectively. Fourier transform infrared (FTIR) spectra were recorded on a PerkinElmer, Spectrum BX II spectrophotometer. The surface topology of the electrodes was studied using atomic force microscopy (AFM) (Veeco DICP2) under the tapping mode. All measurements were carried out at 25 °C.

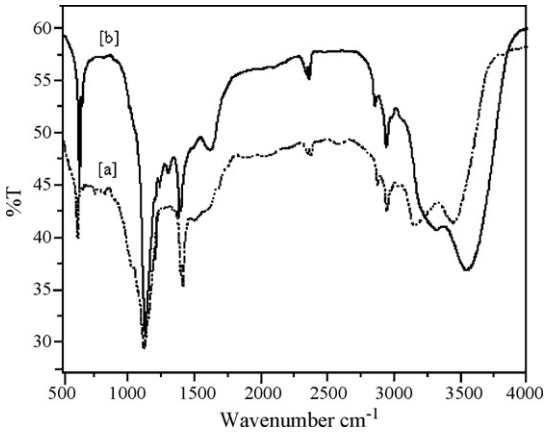
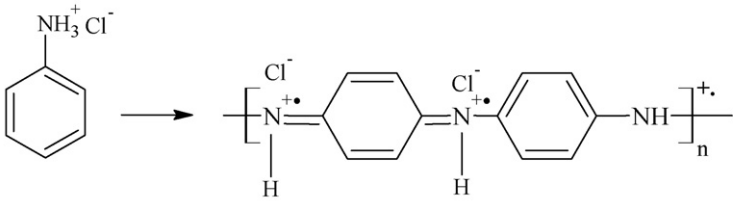


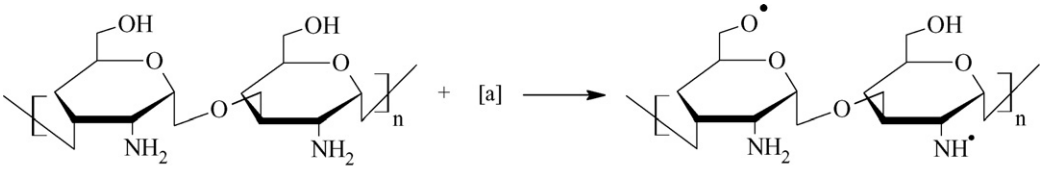
Fig. 2. FTIR spectra of the (a) CHIT-*co*-PANI/ITO and (b) cDNA/CHIT-*co*-PANI/ITO electrodes.

3. Results and discussion

During the electrochemical copolymerization, the aniline monomer initially became protonate with HCl and propagated to form an intermediate called PANI radical cation [30].



PANI radical cation simultaneously generated CHIT macro radicals by the abstraction of hydrogen from the –OH and $–NH_2$ groups of the CHIT macromolecules [21].



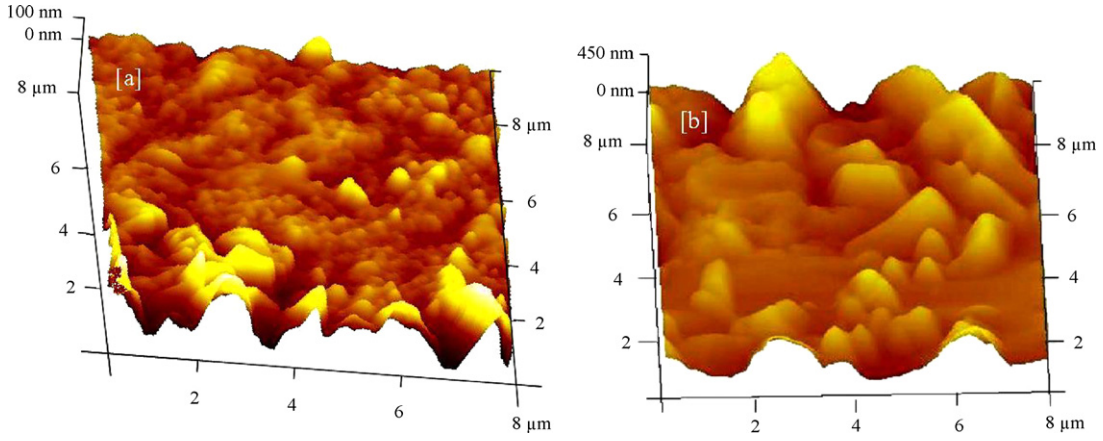
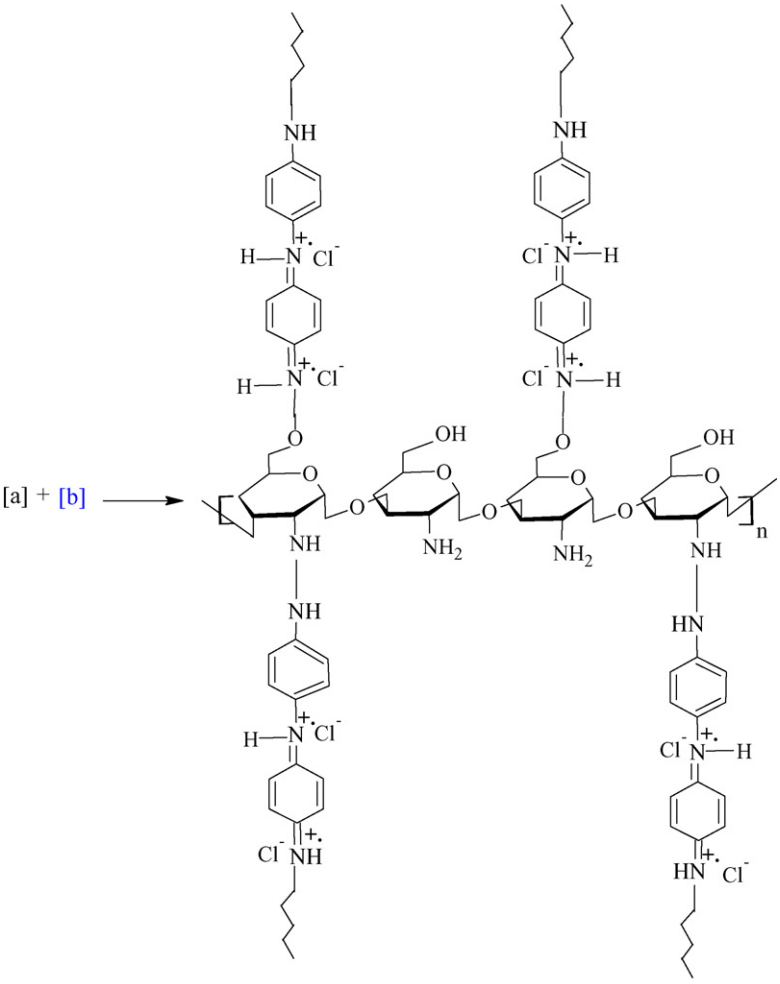


Fig. 3. AFM micrographs of the (a) CHIT-*co*-PANI/ITO and (b) cDNA/CHIT-*co*-PANI/ITO electrodes.

The PANI cation radicals and CHIT macro radicals then copolymerized and yielded CHIT-*co*-PANI.



CHIT-co-PANI

The FTIR spectrum of the CHIT-*co*-PANI/ITO electrode (Fig. 2a) illustrated the characteristic peaks of PANI, as well as CHIT [21]. The following key characteristic bands were observed: (1) 3110–3532 cm^{-1} (free O—H stretching and N—H stretching with hydrogen bonded secondary amino groups); (2) 3023 cm^{-1} (aromatic C—H stretching); 2926 and 2865 cm^{-1} (aliphatic C—H stretching); (3) 1636 cm^{-1} (C=O stretching of carbonyl group, typical saccharide absorption); (4) 1588 cm^{-1} (C=C stretching of quinoid rings); (5) 1489 cm^{-1} (C=C stretching vibration of benzenoid rings); and (6) 1248 cm^{-1} (C—N stretching).

The absorption band of the N=Q=N bending vibration of protonated pure PANI was observed at 1238 cm^{-1}, but shifted to 1121 cm^{-1} in the CHIT-*co*-PANI copolymer due to the steric effect of CHIT [21]. The FTIR spectrum of the cDNA/CHIT-*co*-PANI/ITO electrode (Fig. 2b) showed phosphate vibration at 1226 cm^{-1} (accredited from phosphate backbone of immobilized cDNA) with the peaks broadening at (1) 3105–3364 cm^{-1} (addition of N—H stretching vibration); (2) 2922–2863 cm^{-1}; and (3) 1632–1668 cm^{-1} due to the attachment of cDNA with the CHIT-*co*-PANI matrix. Hence, FTIR spectra confirmed the immobilization of cDNA onto the CHIT-*co*-PANI/ITO electrode.

The surface morphology of the electrodes was observed with AFM and is shown in Fig. 3. Both electrodes exhibited a relatively rough surface topology, which may facilitate the immobilization of cDNA in the case of the CHIT-*co*-PANI/ITO electrode and the hybridization of the target ssDNA in the case of cDNA/CHIT-*co*-PANI/ITO electrode.

Fig. 4 shows the electrochemical characteristics of bare, cDNA immobilized, and DNA hybridized CHIT-*co*-PANI/ITO electrodes. The CHIT-*co*-PANI/ITO electrodes illustrated admirable electrochemical performance. It was observed that the CHIT-*co*-

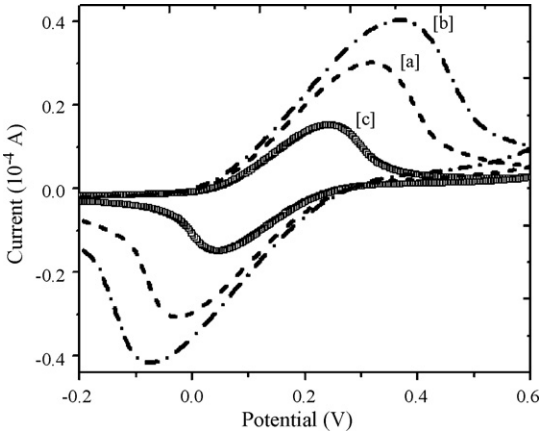


Fig. 4. Cyclic voltammograms of the (a) CHIT-*co*-PANI/ITO; (b); cDNA/CHIT-*co*-PANI/ITO and (c) DNA hybridized CHIT-*co*-PANI/ITO electrodes in PBS (50 mM, pH 7.0, and 5 mM $Fe(CN)_6^{3-/4-}$) at 30 mV s^{-1} scan rate.

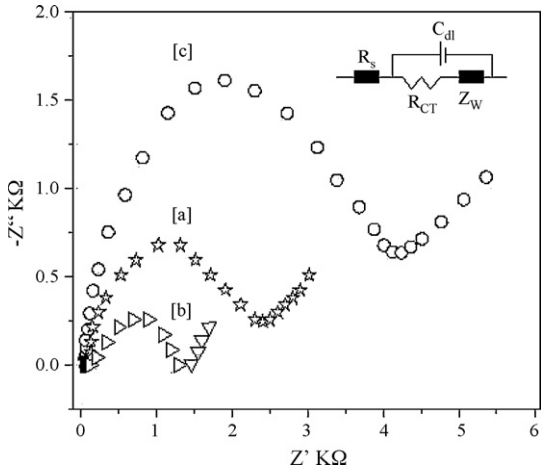


Fig. 5. EIS of the (a) CHIT-*co*-PANI/ITO; (b) cDNA/CHIT-*co*-PANI/ITO; and (c) DNA hybridized CHIT-*co*-PANI/ITO electrodes in PBS (50 mM, pH 7.0, and 5 mM $Fe(CN)_6^{3-/4-}$).

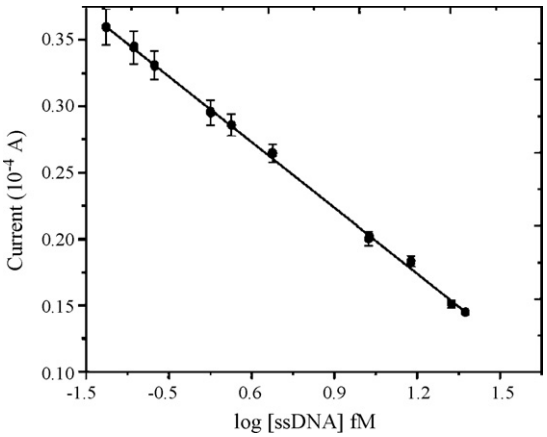


Fig. 6. Steady-state current dependence calibration curve of the biosensor (cDNA/CHIT-*co*-PANI/ITO) to logarithm of molar concentration of the single-stranded target DNA. Working conditions: supporting electrolyte: PBS 50 mM, pH 7.0, and 5 mM $Fe(CN)_6^{3-/4-}$ at potential 0.32 V vs. Ag/AgCl.

PANI/ITO electrodes could enhance the electrochemical response of $Fe(CN)_6^{3-/4-}$, which could be attributed to PANI's excellent electronic conductivity and CHIT's strong ability to interact with the negatively charged $Fe(CN)_6^{3-/4-}$ ions (due to the presence of $-NH_2$ groups in CHIT) [31]. Among the three types of electrodes investigated, the peak current of the cDNA/CHIT-*co*-PANI/ITO electrode was the highest while that of the DNA hybridized CHIT-*co*-PANI/ITO electrode was the lowest. The increase in the peak current observed with the cDNA/CHIT-*co*-PANI/ITO electrode was attributed to the prompt redox behavior of cDNA [32]. The decrease in the peak current measured with the DNA hybridized CHIT-*co*-PANI/ITO electrode may be related to the electrostatic repulsion between the polyanionic hybridized DNA on the CHIT-*co*-PANI/ITO electrode and the anionic redox couple ions [33].

Electrochemical impedance spectroscopy (EIS) is an excellent technique to study the interfacial properties of surface modified electrodes [34,35]. In EIS, the total impedance is measured by various parameters, including solution resistance (R_s), double layer capacitance (C_{dl}), charge transfer resistance (R_{CT}) and Warburg coefficient (Z_w). The R_s and Z_w are usually not affected by the biochemical reactions that occur at the electrode interface; however, both C_{dl} and R_{CT} vary with the interfacial electron-transfer resistances at the electrode–solution interfaces [36]. Fig. 5 shows EIS as Nyquist plot ($-Z'$ vs. Z') of the CHIT-*co*-PANI, cDNA/CHIT-*co*-PANI, and ssDNA hybridized CHIT-*co*-PANI electrodes using 5 mM $[Fe(CN)_6]^{4-/3-}$ as marker ions at EIS frequency from 0.1 Hz to 10 kHz. The Nyquist plot consisted of two steps: first a semicircle and then a straight line. The semicircle diameter of the EIS spectra provides the R_{CT} value that reveals the electron-transfer kinetics of the redox probe at the electrode interface, while the straight line is a typical characteristic of a diffusion limit step. The R_{CT} values for the CHIT-*co*-PANI, cDNA/CHIT-*co*-PANI, and ssDNA hybridized cDNA/CHIT-*co*-PANI electrodes were 2.41 kΩ (curve a), 1.37 kΩ (curve b), and 4.16 kΩ (curve c), respectively. The relatively low R_{CT} value observed with the CHIT-*co*-PANI electrode indicates that the CHIT-*co*-PANI copolymer matrix could have an excellent ability to carry the charge over the electrode surface. In addition, the lower R_{CT} value observed with the cDNA/CHIT-*co*-PANI electrode indicates that immobilizing cDNA on the CHIT-*co*-PANI/ITO electrode enhanced the electron transfer between the electrode and solution.

Fig. 6 shows the calibration curve of the peak current measured with CV using the cDNA/CHIT-*co*-PANI/ITO electrode at varying molar concentrations of the ssDNA ranging from 0.05 to 25 fmol. It was found that the peak oxidation current observed at about 0.32 V decreased with an increasing concentration of the target ssDNA until 25 fmol, beyond which the peak oxidation current remained as a constant. In fact, as shown in Fig. 6, the peak oxidation current decreased linearly with an increasing logarithm of ssDNA molar concentration within the range of 0.05–25 fmol. This result indicates that binding of cDNA with the ssDNA at the cDNA/CHIT-*co*-PANI/ITO electrode surface had occurred. Moreover, it shows that 25 fmol of ssDNA is sufficient to saturate the cDNA immobilized CHIT-*co*-PANI/ITO electrode surface, and 0.05 fmol was detection limit of the biosensor. The sensitivity of the cDNA/CHIT-*co*-PANI/ITO bioelectrode measured was 2.104 μA/fmol and it responded in 16 s. In addition, the surface concentration of the ionic species on the cDNA/CHIT-*co*-PANI/ITO was 6.072 mmol/cm^2, calculated by measuring the oxidation current of CV at different scan rates.

The selectivity of the biosensor was investigated by the hybridization of the cDNA immobilized CHIT-*co*-PANI/ITO bioelectrode with one-base-mismatch ssDNA sequences. After incubation with the mismatched ssDNA sequence, the bioelectrode showed a negligible decrease in the peak oxidation current at 0.32 V, indicating that the biosensor had good selectivity only for the ssDNA.

The reproducibility of the biosensor was measured with a 15 fmol ssDNA sequence. The response currents with seven cDNA/CHIT-*co*-PANI/ITO electrodes fabricated under similar conditions were 2.78, 2.80, 2.78, 2.76, 2.80, 2.81 and 2.78 ($\times 10^{-4}$ A). The seven cDNA/CHIT-*co*-PANI/ITO electrodes had an average current response of 2.79×10^{-4} A with a standard deviation of ±0.02. Thus, the biosensor showed excellent reproducibility.

The shelf life of the cDNA/CHIT-*co*-PANI/ITO bioelectrode was determined by measuring the current at an interval of seven days for up to six months. The amperometric current decreased slowly with an increase in time. The decrease in current measured about 5% after the cDNA/CHIT-*co*-PANI/ITO electrode was stored at room temperature for six months.

4. Conclusions

The cDNA of the breast cancer susceptible gene (BRCA1) was immobilized onto the CHIT-*co*-PANI/ITO electrode. The cDNA/CHIT-*co*-PANI/ITO bioelectrode was employed for the hybridization of the ssDNA, which had a detection limit of 0.05 fmol and showed an excellent sensitivity and reproducibility. The voltammetric sensitivity of the biosensor was 2.104 μA/fmol. In the presence of one-base-mismatched DNA, the amperometric response of the

cDNA/CHIT-*co*-PANI/ITO bioelectrode for the ssDNA was barely affected. Current efforts aim to exploit the cDNA/CHIT-*co*-PANI/ITO electrode for the efficient and precise detection of breast carcinoma at its early stage.

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