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Labelless impedance immunosensor based on polypyrrole–pyrolecarboxylic acid copolymer for hCG detection

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ABSTRACT

In this work, a sensitive label-free impedimetric hCG-immunosensor was constructed by using a commercial screen-printing carbon ink electrode (namely disposable electrochemical printed chip) as the basis. The carbon ink electrode of DEP chip is modified first by deposition of polypyrrole–pyrole-2-carboxylic acid copolymer and thence hCG antibody immobilization via the COOH groups of pyrrole-2-carboxylic acid, which can serve as a linker for covalent biomolecular immobilization. The experimental results exposed that the designed immunosensor is more sensitive than other previously reported immunosensors, in the case of detection limit and linear range for antigen detection. With optimal fabrication parameters, the detection limit for α-hCG was 2.3 pg/mL in 10 mM phosphate buffer saline (PBS) solution containing 1% bovine serum albumine (BSA). Moreover, the use of inexpensive DEP chip as a basis for these immunosensors will allow for simple instrumentation, disposable and portable at low cost. This work also demonstrates a new approach to develop a sensitive and label-free impedimetric immunosensor based on screen-printed electrode for applications in clinical diagnosis.

1. Introduction

Impedance biosensors are now attracting considerable attention in the many areas of bioanalytical chemistry, analysis mutant genes, clinical diagnosis for health care and diagnosis of infectious agents in various environments because of their advantages over number of electrochemical biosensors such as amperometric and potentiometric. This sensor is label-free with a direct detection of the specific binding event, less destructive to the activities of the bioentities due to the small voltage excitation during detection, simple operation and very sensitive with comparable detection limits as optical-based sensor [1–3]. However, the immobilization of biological molecules is decisive factor for successful fabrication of biosensors. The immobilization procedure must maintain the activity as well as enhance stability of biomolecules and give some control over the distribution and orientation of the immobilized species.

Conducting polymers such as polyacetylene, polyaniline and polypyrrole have been extensively studied for immobilization of biomolecules applications. Polypyrrole is one of the most conducting polymers used because of its good biocompatibility [4], simply synthesis and can be modified the required electronic as well as mechanical properties by chemical modeling and synthesis [5,6]. Moreover, the polypyrrole thin film can directly be deposited over defined areas of different electrode substrates using electrochemical method followed by biomolecules immobilization [7,8]. Therefore, it is possible to control the spatial distribution of the immobilized biomolecules as well as the film thickness.

The human chorionic gonadotropin (hCG) is glycoprotein composed of 244 amino acids with a molecular mass of 36.7 kDa [9]. Its most important uses as a tumor marker are in gestational trophoblastic disease and germ cell tumors. Measurement of hCG is important in establishing the diagnosis of the disease.

In this work, we describe the development and characterization of label-free impedance immunosensor for hCG detection. The sensor employs commercial disposable electrochemical printed (DEP) chip as the basis. The carbon ink electrode of DEP chip is modified first by deposition of polypyrrole–pyrole-2-carboxylic acid copolymer and thence hCG antibody immobilization via the COOH groups of pyrrole-2-carboxylic acid, which can serve as a linker for covalent biomolecular immobilization.

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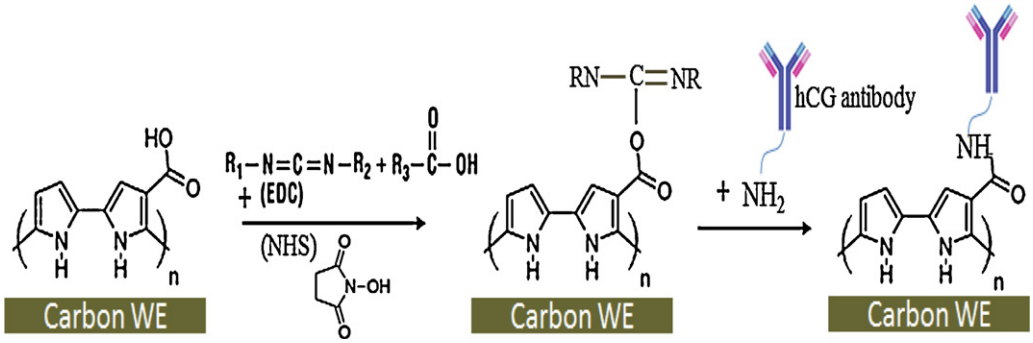

Fig. 1. Schematic diagram representation of the covalent attachment of hCG antibodies to the activation carboxyl-terminated PPy–PPa copolymer layer using EDC hydrochloride and NHS.

2. Experimental

2.1. Reagents and apparatus

The following materials as indicated: pyrrole (Py) monomer, pyrrole-2-carboxylic acid (Pa) and ethanolamine were purchased from Sigma–Aldrich. The hCG monoclonal antibody (Mab) and antigen were supplied by Medix Biochemica (Finland). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) hydrochloride was supplied from Dojindo (USA). N-hydroxysuccinimide (NHS) and Tween 20 were obtained from Wako. All reagents used were of the analytical grade or the highest commercially available purity and used as supplied without further purification. All solutions were prepared with deionized water of resistivity no less than 18 MΩ cm. The commercial DEP chips containing carbon ink working, carbon ink counter and Ag/AgCl ink reference electrodes were obtained from BioDevice Technology Ltd., Japan (http://www.biodevicetech.com). The surface area of the working electrode is 2.64 mm^2. AutoLab PGSTAT 30 system (EcoChemie B.V., Ultrecht, The Netherlands) was used to conduct cyclic voltammetry in order to deposit polymer film onto carbon ink working electrode and perform electrochemical impedance spectroscopy (EIS) measurements.

2.2. hCG immobilization procedure

Cyclic voltammetry was used to deposit carboxyl-terminated polypyrrole (PPy–PPa) film onto the carbon ink working electrode. The DEP chips were immersed into a solution containing 120 mM Py, 100 mM KCl and 40 mM Pa (the solution was purged with nitrogen gas for 15 min before use in order to remove dissolved oxygen) and cycled from −800 mV to +800 mV versus Ag/AgCl for 20 cycles at scan rate of 20 mV/s. At these polymerization conditions it is found that the same quality of PPy–PPa film was deposited on each electrode. Following deposition, the copolymer-coated electrodes were washed several times in 10 mM phosphate buffer saline (PBS) solution containing 0.05% Tween 20 followed by deionized water and then dried under nitrogen (N_2) stream. This copolymer-coated electrode is ready to immobilize hCG antibody (Fig. 1).

Before hCG antibody immobilization, the terminal carboxylic groups of copolymer PPy–PPa films were activated by NHS–EDC hydrochloride. 0.2 M EDC hydrochloride and 0.1 M NHS solution were prepared in deionized water. A volume of 2 μL of the solution was dropped onto the copolymer-coated working electrode surface for 15 min at room temperature. In this step, the EDC hydrochloride converts carboxyl group into amine-reactive *O*-acylisourea intermediate, which is susceptible to attack by amines lysine residues of antibody and amide bonds then form between the antibody molecules and carboxyl-terminated monolayers. However, the intermediate is also susceptible to hydrolysis, making it unstable and short-hydrolysis in aqueous solution. The addition of NHS stabilizes the amine-reactive intermediate by converting it to an amine-reactive NHS ester, thus increasing the efficiency of EDC-mediated coupling reactions [10,11]. The amine-reactive NHS ester intermediate has sufficient stability to permit two-step crosslinking procedures, which allows the carboxyl groups on one protein to remain unaltered. After activation, the electrodes were rinsed with 10 mL of 10 mM PBS solution containing 0.05% Tween 20 followed by deionized water and dried under a gentle stream N_2 gas. Then, 2 μL of 100 μg/mL Mab hCG solution was placed onto activated-PPy modified electrode for 1 h at room temperature. In this procedure, Mab hCG was success immobilized onto the PPy–PPa film. Following this, the electrode was washed with 10 mL of 10 mM PBS solution containing 0.05% Tween 20 followed by deionized water to remove the loosely bound antibodies and dried over a stream N_2 gas. Finally, the Mab hCG-modified electrode was subjected to 2 μL of 100 mM ethanolamine solution for 1 h at room temperature in order to block the remaining non-specific adsorption-reactive sites. The electrode was also rinsed with 10 mL of 10 mM PBS solution containing 0.05% Tween 20 followed by deionized water, then dried over a gentle stream N_2 gas and used immediately. Fig. 1 shows the whole activation reaction process.

2.3. hCG antigen detection procedure

The hCG antigens were suspended in 10 mM PBS solution containing 1% bovin serum albumine (BSA) with required antigen

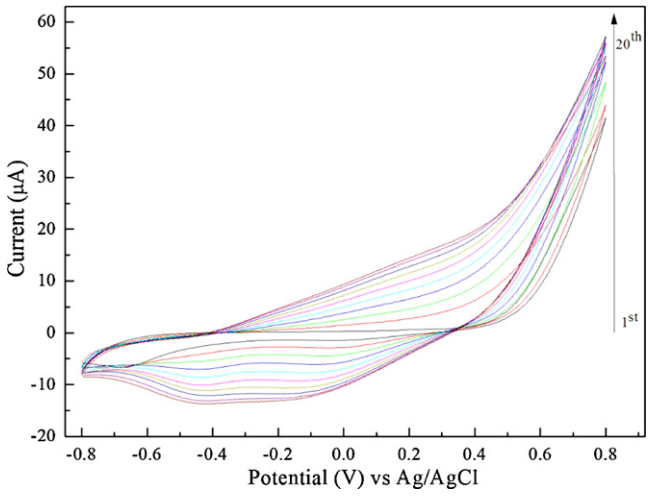

Fig. 2. Repetitive cyclic voltammograms recorded during the electropolymerization of PPy–PPa copolymer film. Solution: Py 120 mM, KCl 100 mM and Pa 40 mM. Scan rate: 20 mV/s.

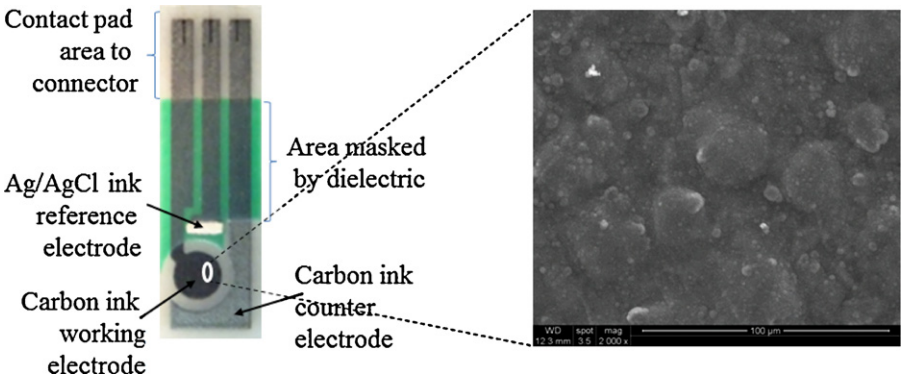

Fig. 3. The structure of disposable electrochemical printed (DEP) chip used within this work and SEM micrograph of deposited PPy–PPa copolymer film onto carbon ink working electrode.

concentration. In this case, the range of hCG antigen concentration from 100 fg/mL to 40 ng/mL was utilized.

The sensors were first EIS measured without antigen addition. Following this, 2 μL required antigen concentration was added on each sensor surface for 30 min at room temperature to let the antigens attach to the antibodies. Then, sensors were rinsed with 10 mM PBS followed by deionized water and dried over a gentle stream N_2 gas. Finally, all sensors were subjected to EIS measurement. The impedance spectra was recorded in 0.1 M KCl solution containing 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ within the frequency range from 100 kHz to 50 mHz. An ac probe amplitude of 10 mV was applied to the system around the open circuit potential (OCP).

3. Results and discussion

3.1. PPy–PPa composite film formation

Based on the nature of PPy and PPa, we recognize that PPy is conductive but hydrophobic, while PPa is less conductive but very hydrophilic. For this reason, it is very necessary to optimize the ratio of Py to Pa in order to have a composite film which is designed for highly sensitive and stable impedance biosensor. This film could take advantage from the high conductivity and good biocompatibility of PPy, the enhanced hydrophilicity and covalent binding ability of PPa. The ratio of Py to Pa in this composite film was accommodated by changing the ratio of Py to Pa in the deposition solution. In this work, the Py/Pa ratios were 1:1, 2:1 and 3:1, respectively. The composite films produced with ratio of 3:1 show the best electroactivity and provide a high concentration of COOH groups (data not shown). This result was consistent with report of Chen et al. [12]. This report indicates that the Py percentage in (Py + Pa) should be higher than 70% to have the highest density of COOH group while producing the excellent electroactivity. Therefore, the impedance biosensor used within this work was fabricated from PPy–PPa film deposited with Py/Pa ratio of 3:1. The cyclic voltammograms for the deposition of PPy–PPa copolymer film onto the carbon ink working electrode of DEP chip are represented in Fig. 2. The increase in current from scan 1st to 20th is due to the increase in composite thickness and coverage of the electrode. Fig. 3 shows the structure of the DEP chip with copolymer film-modified electrode.

3.2. Labelless impedance immunosensor

In impedance immunosensor, the detection is based on the principle that any substance attached on the electrode will change the measured impedance. In this case, the hCG antibody receptor and the bound hCG antigen together can be considered as a coating film which is expected to affect the sensor impedance signal. The impedance measurement can be performed in the absence or presence of a redox probe, which are referred to nonfaradaic and faradaic impedance measurements [13]. In the absence of a redox probe, the measured impedance signal results directly from the substances that are adherently attached to the electrode surface. In other words, the impedance is influenced with the changes in amount, growth and morphological behavior of adherent substance. In the presence of a redox probe, the sensor verifies the biological events occurring on its surface by measuring the changes in impedance spectroscopy. Therefore, this method has been considered as an efficient way to monitor the formation of antigen–antibody interaction. In this work, we developed a electrochemical impedance immunosensor using a redox probe, $[Fe(CN)_6]^{3-/4-}$, for α-hCG detection.

Fig. 4 shows the impedance responses for each step in the stepwise modification of electrodes. The impedance spectra include a semicircle part at high frequency region corresponding to the electron transfer limited process and a linear part at lower frequencies resulting from the diffusion limiting step of the electrochemical process. Significant difference in the impedance spectra of Mab hCG immobilization-modified electrode and binding of hCG antigen compared with bare electrode were observed. The diameter of semicircle equals to electron transfer resistance, which denotes the blocking behavior of the electrode surface for redox couple. Therefore, the increase and decrease in this value will be exhibited

Table 1
Impedance parameters were obtained from the Randle's equivalent circuit fit to the impedance spectra which is presented in Fig. 5.

hCG (pg/mL)	Randles circuit elements		
	R_{CT} (kΩ)	C_{dl} (μF)	R_s (kΩ)
Blank	9.63 ± 1.01	0.25 ± 0.03	8.60 ± 1.07
0.1	11.65 ± 1.02	0.39 ± 0.05	8.55 ± 1.02
1.0	12.13 ± 1.42	0.38 ± 0.03	8.56 ± 0.95
10	12.67 ± 1.92	0.41 ± 0.02	8.64 ± 1.04
100	14.04 ± 2.26	0.40 ± 0.02	8.64 ± 1.07
1000	15.38 ± 2.18	0.40 ± 0.02	8.65 ± 1.08
2000	15.82 ± 2.25	0.34 ± 0.03	8.68 ± 1.08
3000	16.94 ± 1.51	0.35 ± 0.03	8.66 ± 1.04
4000	18.51 ± 2.25	0.35 ± 0.02	8.60 ± 1.00
5000	19.74 ± 2.13	0.34 ± 0.04	8.65 ± 1.04
6000	19.85 ± 1.58	0.34 ± 0.01	8.68 ± 1.03
7000	20.17 ± 1.77	0.43 ± 0.04	8.68 ± 1.12
8000	21.45 ± 0.85	0.35 ± 0.03	8.70 ± 1.10
9000	23.83 ± 0.35	0.28 ± 0.04	8.88 ± 1.15
10,000	26.46 ± 2.11	0.30 ± 0.03	8.65 ± 1.08
15,000	27.78 ± 1.98	0.32 ± 0.04	8.68 ± 1.10
20,000	27.47 ± 3.21	0.29 ± 0.04	8.67 ± 1.07
30,000	28.45 ± 3.12	0.32 ± 0.04	8.65 ± 1.08
40,000	29.97 ± 1.45	0.29 ± 0.03	8.68 ± 1.05

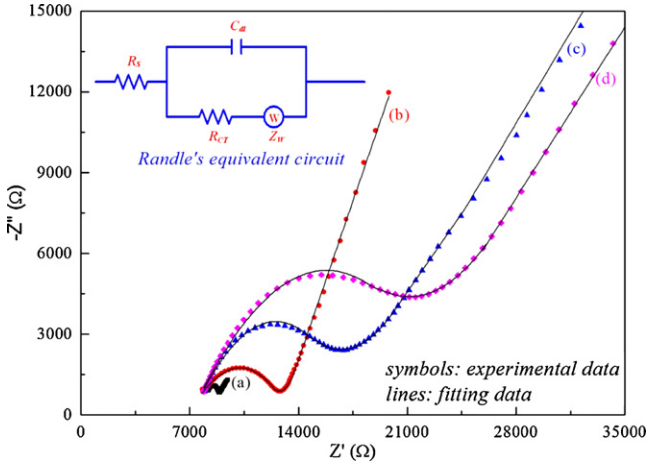

Fig. 4. Schematic showing the Randle's equivalent circuit to fit impedance spectroscopy in the presence of the redox couple of $[Fe(CN)_6]^{3-/4-}$ and impedance changes during stepwise modification of the electrode: (a) carbon bare electrode; (b) PPy–PPa copolymer modified electrode; (c) Mab hCG immobilization onto copolymer modified electrode and (d) the hCG antibody–antigen interaction occurs on modified electrode surface. Symbols show experimental data and lines show the fitting data using commercial software Autolab data analysis (EcoChemie).

exactly the assembly of electrode surface. The impedance data were fitted with commercial software Autolab data analysis (EcoChemie) using the Randle's equivalent circuit (see Fig. 4). The circuit includes the following four elements: (1) the solution-phase resistant R_s; (2)

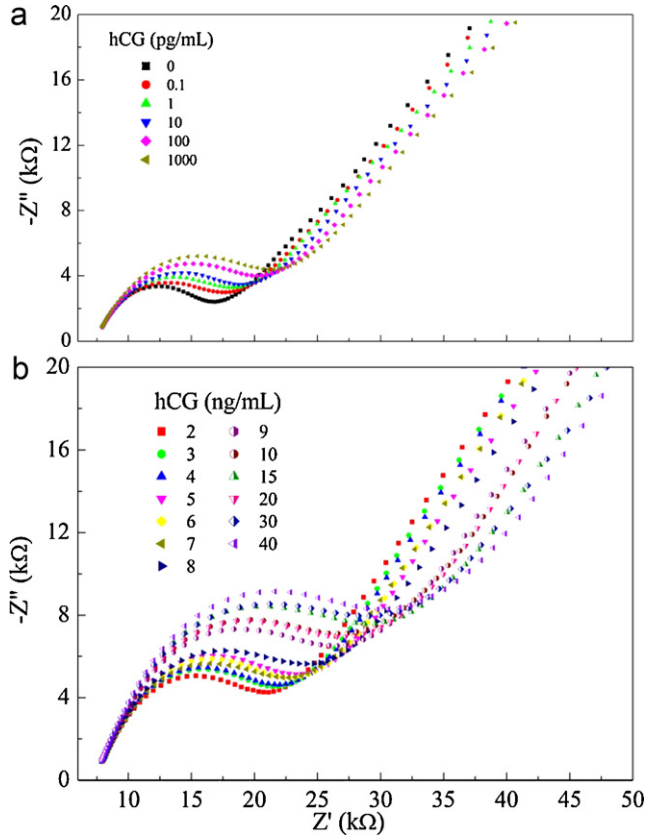

Fig. 5. Nyquist plots obtained on the Mab hCG-copolymer modified electrodes after treatment of the different concentrations of hCG antigen (a) from 0 pg/mL to 1000 pg/mL and (b) from 2 ng/mL to 40 ng/mL in the solution containing 0.1 M KCl and 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$. The impedance results were obtained at OCP and frequency range is from 100 kHz to 50 mHz with an ac probe amplitude of 10 mV.

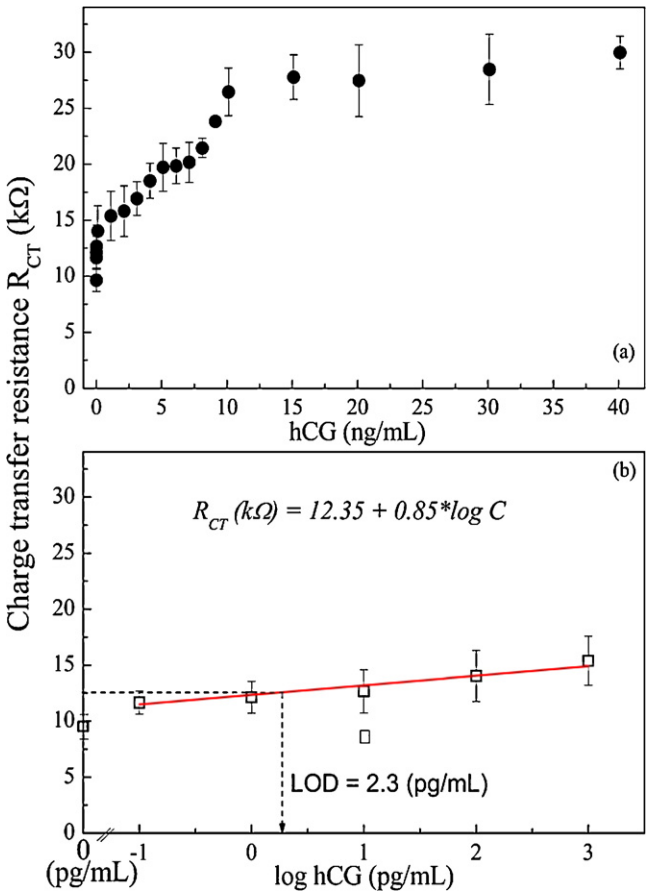

Fig. 6. The calibration curve obtained the Mab hCG-copolymer modified electrodes using R_{CT} as function of hCG antigen concentration C. All data points are average values for the responses of three electrodes and error bars give a measure of the reproducibility of the system.

the Warburg impedance Z_W; (3) the double layer capacitance C_{dl}; and (4) charge transfer resistance R_{CT}. Ideally, the Z_W and R_s represent the properties of the electrolyte solution and diffusion of the redox probe, thus, they are not affected by modification occurring on the electrode surface [8,14]. So, there are only two parameters, C_{dl} and R_{CT}, that are mainly used in impedance sensor. In this case, R_{CT} was used as a signal for sensing the interfacial properties of electrodes, using redox couples $[Fe(CN)_6]^{3-/4-}$ in the solution. For the carbon bare electrode, the value of R_{CT} is 0.72 ± 0.02 kΩ. However, the forming of PPy–PPa copolymer film on carbon electrode acts as an insulating layer which obstructs interfacial charge transfer and therefore the diameter of semicircle drastically increases with an increase in R_{CT} value to 5.88 ± 0.29 kΩ. A noteworthy increase in R_{CT} value to 9.63 ± 1.01 kΩ was observed in the step of hCG antibody immobilization. An increase in the charge transfer resistance value could be explained due to the generation of an insulating protein layer on electrode. This result was confirmed that the hCG monoclonal antibody was successfully immobilized onto PPy–PPa copolymer film. Following this, the hCG immobilization modified electrode was exposed to hCG antigen with concentration of 1 ng/mL and an increase in the value of R_{CT} to 15.38 ± 2.18 kΩ was observed. This result was further confirmed the success of hCG antibody immobilization on the electrode.

3.3. Impedance spectra of hCG antibody–antigen interactions

To evaluate the interaction between hCG antibody and antigen, the hCG antibody-copolymer modified electrodes are exposed

to various concentration of hCG antigen (from 100 pg/mL to 40 ng/mL). The corresponding Nyquist plots of impedance spectra are shown in Fig. 5 and the fitting impedance parameters are presented in Table 1. The results shown that the diameter of the Nyquist semicircle increases with increasing of hCG antigen. This could be due to the binding of more antigen molecules to immobilized hCG antibody in higher concentration of antigen. Therefore, the interfacial charge transfer was hindered significantly, resulting in a corresponding increase in the charge transfer resistance. The increase in the R_{CT} with increasing protein coverage has been reported [8,15–17]. Furthermore, the relative change in R_{CT} is much larger than the relative change in C_{dl} during antigen binding. This is often observed for impedance protein detection [2,16,17].

The calibration curve was obtained by plotting the relative R_{CT} versus antigen concentration and shown in Fig. 6. As can be seen, R_{CT} increased very fast with increasing of antigen concentration within the detected concentration in the range of 100 pg/mL–1 ng/mL and slowdown in range of 2 –10 ng/mL. However, the increase in R_{CT} was not observed at higher concentration of antigen (from 20 ng/mL to 40 ng/mL) due to saturation of coupled antigen molecules. Based on that the linear range was obtained from 100 pg/mL to 1 ng/mL with the linear equation of R_{CT} ($k\Omega$) = 12.35 + 0.85 × log C (R^2 = 0.93), the detection limit for hCG of this sensor was determined to be 2.3 pg/mL with the sensor area of 2.64 mm^2. This result shows that the designed immunosensor is more sensitive than the other previously reported impedimetric immunosensor, in the case of detection limit and linear range for antigen detection [8,17–20].

4. Conclusion

The results presented in this work concern successful implementation of the EIS to demonstrate an impedance biosensor for detection of hCG antigen which is responsible for the gestational trophoblastic tumor marker diagnosis. The results indicated that PPy–PPa copolymer film deposited on the carbon ink working electrode of DEP chip provided a highly active surface for the immobilization of hCG antibody molecules with maintained immunoactivity. Additional information, the experimental results exposed that the designed sensor is more sensitive than the other previously reported immunosensor, in the case of detection limit and linear range for antigen detection. Moreover, the use of inexpensive DEP chip as a basis for these sensors will allow for simple instrumentation, disposable and portable at low cost. Besides, based on the current study, it was found that EIS is an impressive method for monitoring the interaction of antigen with antibody that occurred on the electrode surface. This method used in this work could also be applied to detect other antigens.

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