



Multi-wall carbon nanotubes (MWCNTs)-doped polypyrrole DNA biosensor for label-free detection of genetically modified organisms by QCM and EIS

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ABSTRACT

In this paper, we describe DNA electrochemical detection for genetically modified organism (GMO) based on multi-wall carbon nanotubes (MWCNTs)-doped polypyrrole (PPy). DNA hybridization is studied by quartz crystal microbalance (QCM) and electrochemical impedance spectroscopy (EIS). An increase in DNA complementary target concentration results in a decrease in the faradic charge transfer resistance (R_{ct}) and signifying "signal-on" behavior of MWCNTs-PPy-DNA system. QCM and EIS data indicated that the electroanalytical MWCNTs-PPy films were highly sensitive (as low as 4 pM of target can be detected with QCM technique). In principle, this system can be suitable not only for DNA but also for protein biosensor construction.

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1. Introduction

In recent years, there has been considerable interest in the development of DNA sensors thanks to their numerous applications such as the analysis of unknown or mutant genes, diagnosis of infectious agents in various environments and detection of analytes (drugs, pollutants, etc.). Traditional methods for DNA detection, based on the radioisotopic and fluorescent detection, are labor and time consuming, and are, thus, not well suited for routine and rapid medical analyses, particularly for point-of-care tasks. Among some new approaches to DNA detection, electrochemical detection has many advantages such as the reduction of the assay time, simple protocol and therefore can be used for on-site monitoring. In this context, oligonucleotides (ODN, a short fragment of DNA) immobilization at an electrode surface has been used in the most studies [1–4].

Among known conducting polymers, polypyrrole (PPy) is most frequently used in the commercial applications due to the high conductivity, long-term stability of its conductivity and the possibility

of forming homopolymers or composites with optimal mechanical properties. PPy offers potential applications in the domain of composite materials, tissue engineering, actuators, supercapacitors, electronic and electro optic devices [5–9]. Especially, functionalized PPy polymers have attracted great attention in label-free detection because of their surface functionality and electron transduction [10]. On the other hand, carbon nanotubes (CNTs) have attracted considerable studies since their discovery [11]. They display high electrical conductivity, good mechanical strength and excellent chemical stability. The ability of CNTs-modified electrodes to promote electron-transfer reactions has been reported in connection with many important biomolecules [12,13].

Therefore, the combination of conducting polymer with CNTs was a good idea and in reality it was proven to have enhanced charge density, electrical conductivity and electrocatalytic activity compared with each pure component [14–16].

Recently, agricultural enterprises in the world have developed new plant varieties by adopting modern biotechnology, including genetic transformation. In the US, more than 40% of the corn, 50% of the cotton and >45% of soybean acres planted in 1999 have been genetically modified; at least 60% of food production US supermarkets contain genetically modified organisms (GMOs) [17]. A GMO is referred to as a living organism whose genome has been modified by the introduction of an exogenous gene able to express an additional protein that confers new characteristics, i.e. herbicide tolerance [18–20].

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The exogenous DNA that has been integrated into a crop consists of a promoter (P), a structural gene ('encoding region') and a transcription terminator (T). Two particular sequences are inserted in most of the available transgenic products: the promoter of the subunit 35S of ribosomal RNA of *Cauliflower mosaic virus* (35S) and the Terminator of nopaline synthase (NOS) gene from *Agrobacterium tumefaciens*. In particular, they are widely used for the production of many transgenic vegetables commercially available, as Roundup Ready soy, MaisGard maize and the Flavr Savr tomato. Thus, the 35S and NOS sequences are the most frequently used targets for GMO screening in DNA detection approach (this approach is more often used than the second one of protein detection because DNA stability is much higher than that of proteins and therefore DNA detection can be used for both raw and processed materials while protein detection is suitable only for raw materials).

On the need of fast and sensitive analytical methods for GMO screening, in this study, we described the setup of a novel DNA label-free electrochemical biosensor for GMO (soybean) detection, based on MWCNT-doped PPy matrices for ODN immobilization and hybridization.

2. Experimental

2.1. Chemicals

Pyrrole (Py) 99% were purchased from Sigma–Aldrich. It was distilled under reduced pressure before use. Other reagents used were of analytical grade or the highest commercially available purity and used as supplied without further purification. The multi-wall carbon nanotubes (MWCNT, ~95% purity), prepared by the Chemical vapor deposition method, were obtained from Nanolab (China). The diameter of MWCNT is in the range of 40–60 nm and with a length of 1–2 μm . All aqueous solutions were prepared using Milli-Q water (18 M Ω cm).

2.2. Functionalization of MWCNT with carboxylic groups

The functionalization of CNTs has been achieved by an oxidation process, which involves extensive ultrasonic treatment in a mixture of concentrated $\text{H}_2\text{SO}_4/\text{HNO}_3$. The ends and sidewalls of the treated CNTs are mainly decorated with carboxyl groups. CNTs functionalized in this manner obtain good solubility in water and retain their primary electronic and mechanical properties [21].

A calculated amount of MWNTs was added into 3:1 $\text{H}_2\text{SO}_4/\text{HNO}_3$ mixture, then ultrasonicated in a water bath for several hours at 40 °C. The resulting suspension was diluted with deionized (DI) water and was centrifuged. The pretreated MWNTs (functionalized cMWCNT) were then washed with DI water for several times, treated with 0.1 M NaOH (to reach pH of 7), filtered, and dried at 60 °C overnight. The desired amount of the cMWCNTs (3 mg ml⁻¹) was added in DI water and ultrasonicated for about 15 min to form a uniform cMWCNTs black solution.

2.3. ODN sequences

In this study the herbicide-resistance RR soybean (product of Monsanto Inc.) has been chosen as a model. In this genome, promoter sequence is found as the CaMV 35S, which is extracted from *Cauliflower mosaic virus*. The synthesized ODN probe-pyrrole, non-complementary and complementary targets were purchased from Invitrogen. Their sequences were listed in Table 1. The PCR amplification of ODN 35S probe and its complementary target were presented in Fig. 1.

Table 1

Composition of PBS buffer and sequences of used ODN probe and targets (complementary and non-complementary strands).

Materials	Composition/sequence
PBS buffer	0.14 M NaCl; 2.7 mM KCl; 10 mM Na ₃ PO ₄ ; 1.7 mM K ₃ PO ₄ ; pH 7.4
ODN probe (P35S)	5' GGCCATC GTTGAAGATGCCTCTGCC 3'
ODN target CaMV 35S promoter (complementary target)	5' GGCAGAGGCATCTTCAACGATGGCC 3'
2-Base non-complementary target (NC target)	5' GGCAGAGGCACTTCAAGATGGCC 3'

2.4. Electrosynthesis of films

Pure PPy film (denoted as PPy) was electrochemically deposited on the Au microelectrode either at a constant potential and time (+0.7 V vs. Ag/AgCl for 30 min or by voltammetric cycling from 0 to 0.9 V vs. Ag/AgCl for 30 scans, with the scan rate of 20 mV s⁻¹) from electrolytic solution containing PBS buffer, Py (0.3 M).

Probe immobilized PPy films (PPy-ODN) and probe immobilized CNT-doped PPy (C-PPy-ODN) films were obtained with the same procedure as described above with addition of ODN probe (1 $\mu\text{l ml}^{-1}$) and mixture of ODN probe (1 $\mu\text{l ml}^{-1}$) and cMWCNTs (3 mg ml⁻¹), respectively, followed by the action of ultrasonication.

2.5. ODN hybridization

For hybridization experiments, a set of ODN targets (1 $\mu\text{l ml}^{-1}$) was used: CaMV 35S as complementary target, and 2-base muted sequence, non-complementary (NC) target as a control. The hybridization experiments were performed in PBS buffer (pH 7.4) containing target strand for 30 min or 1 h at room temperature followed by an adequate rinse with the same buffer solution.

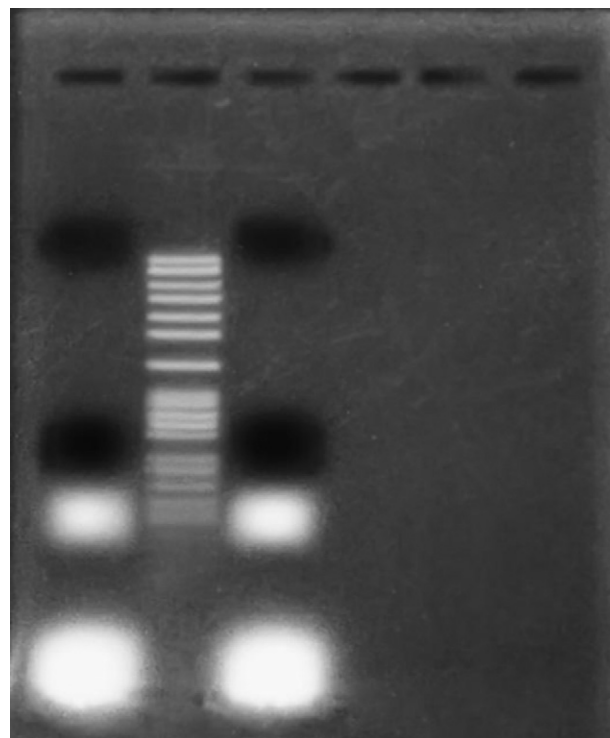


Fig. 1. The amplicon obtained by the PCR of ODN probe (P35S, left band) and its complementary target (right band).

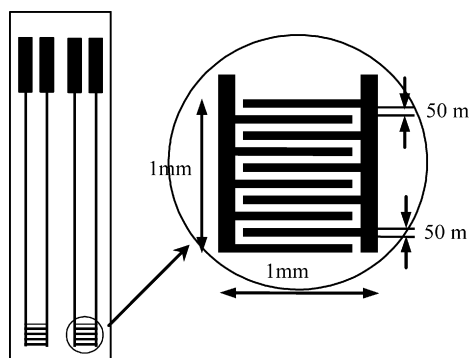


Fig. 2. Schematic representation of working interdigitated microelectrode.

2.6. Quartz crystal microbalance (QCM)

The quartz crystals employed in this study were sputtered AT-cut type (13.67 mm diameter) with Au electrodes (5.1 mm in diameter), nominal frequency (5 MHz) with Stanford QCM 200 frequency analyzer. Frequency variations are related to mass change following the Sauerbrey equation: $\Delta f = -(2/\rho v)f_0^2(\Delta m/S)$. The sensitivity is an experimental value determined by the constructor. ρ is the quartz density and v its frequency coefficient. QCM measurements enable the detection of the variation of attached mass through the modification of the quartz crystal frequency to occur. Under the condition of linear behavior, occurred when the mass variation Δm is lower than some $\mu\text{g cm}^{-2}$, the mass uptake is directly proportional to minus the frequency variation Δf through an experimental factor determined by the following calibration equation: $\Delta m/\Delta f = -1.1 \times 10^{-9} \text{ g Hz}^{-1}$. Regarding the aforementioned sensitivity, the QCM offers the ability to detect the hybridization of the immobilized ODN probes with the ODN targets (Table 2).

2.7. Electrochemical measurements

All electrochemical experiments in this work were performed using Autolab PGSTAT 12 system (EcoChemie B.V., Utrecht, The Netherlands). A conventional three-electrode cell was employed. Working electrode (WE) was either Au or Pt comb-type microelectrodes (Fig. 2) (in electrochemical experiments) or quartz (in QCM experiments). Pt foil was employed as the counter electrode (CE) and Ag/AgCl 3 M KCl was used as a reference electrode (RE). The electrolyte and the monomer (pyrrole) were mixed together to form a solution and then the solution was purged with nitrogen for 10 min to remove oxygen from the solution.

Electrochemical impedance spectroscopy (EIS) measurements were performed in the frequency range 200 kHz to 100 MHz using 5 mV alternating voltage superimposed on DC potential. Before each measurement, the same constant was imposed as a pretreatment for 120 s (Table 3).

Table 2

Frequency variation on C-PPy-ODN films vs. CaMV 35S concentration during hybridization experiments.

CaMV 35S concentration (pM)	Frequency (kHz)	CaMV 35S concentration (pM)	Frequency (kHz)	CaMV 35S concentration (pM)	Frequency (kHz)
0	2803.620	39	2728.606	81	2671.772
4	2798.995	43	2711.519	96	2668.262
9	2794.660	46	2706.566	117	2663.669
13	2790.531	49	2699.414	134	2659.030
17	2787.518	52	2693.783	164	2654.393
21	2784.145	56	2687.886	195	2646.078
25	2780.431	59	2683.734	224	2640.364
28	2776.974	62	2681.768	251	2636.650
32	2771.526	65	2678.836	296	2634.700
36	2746.940	70	2676.459		

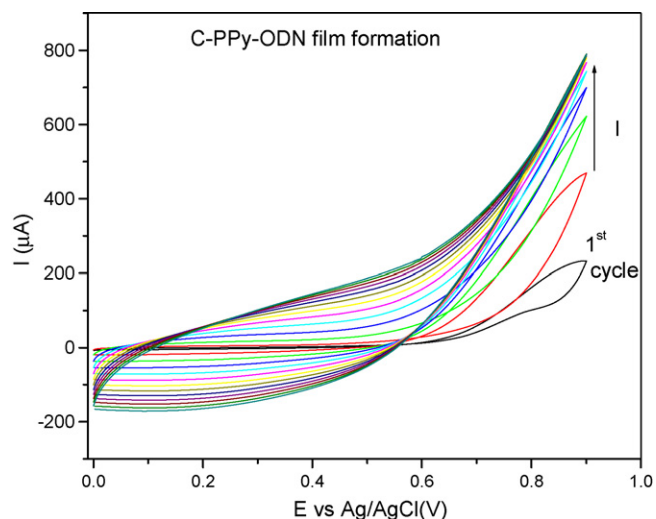
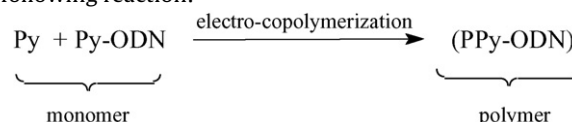


Fig. 3. Cyclic voltammograms during electropolymerization of C-PPy-ODN film.

3. Results and discussion

3.1. Film formation and morphology

Normally, sensitivity and reproducibility of DNA sensors are determined by the surface chemistry of the recognition interface, therefore immobilization of DNA probe on solid surfaces is the primary issue for DNA sensor construction. In our case, ODN probe immobilization was carried out via copolymerization of pure Py monomer with ODN modified Py moieties (Py-ODN) according to the following reaction:



The cyclic voltammograms during film formation were presented in Fig. 3. The WE consists of two electrode pairs, called measuring and reference one, made of Pt thin film, sputtered on a silicon wafer of which the surface was oxidized. The thickness of the metal electrode is about 150 nm. The use of this couple of electrodes is aimed at eliminating the change of charges in solution allowing the improvement of the signal to noise (S/N) ratio of WE. Morphologies of pure PPy, PPy before and after hybridization with ODN were presented in Fig. 4: from left to right are SEM images of pure PPy, PPy-ODN and C-PPy-ODN, respectively.

3.2. Label-free detection of CaMV 35S complementary target by QCM measurements

The hybridization events with complementary and muted targets were detected by QCM and EIS. QCM is known to provide

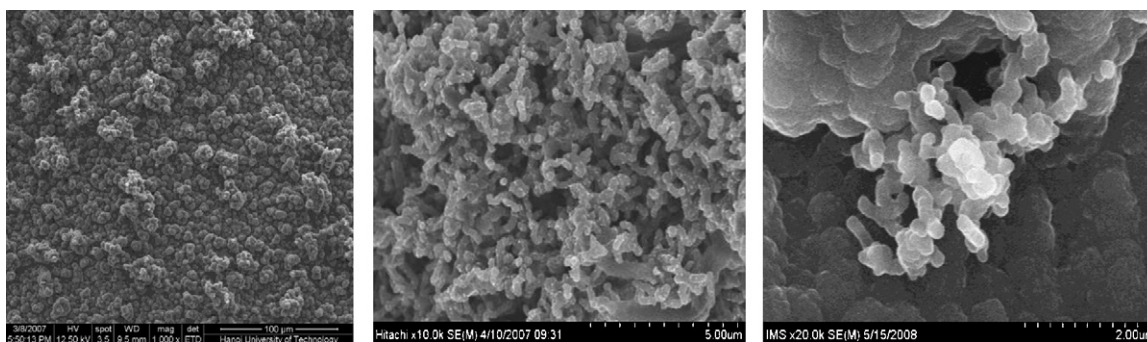


Fig. 4. SEM images of PPy (left), C-PPy (middle) and C-PPy-ODN (right).

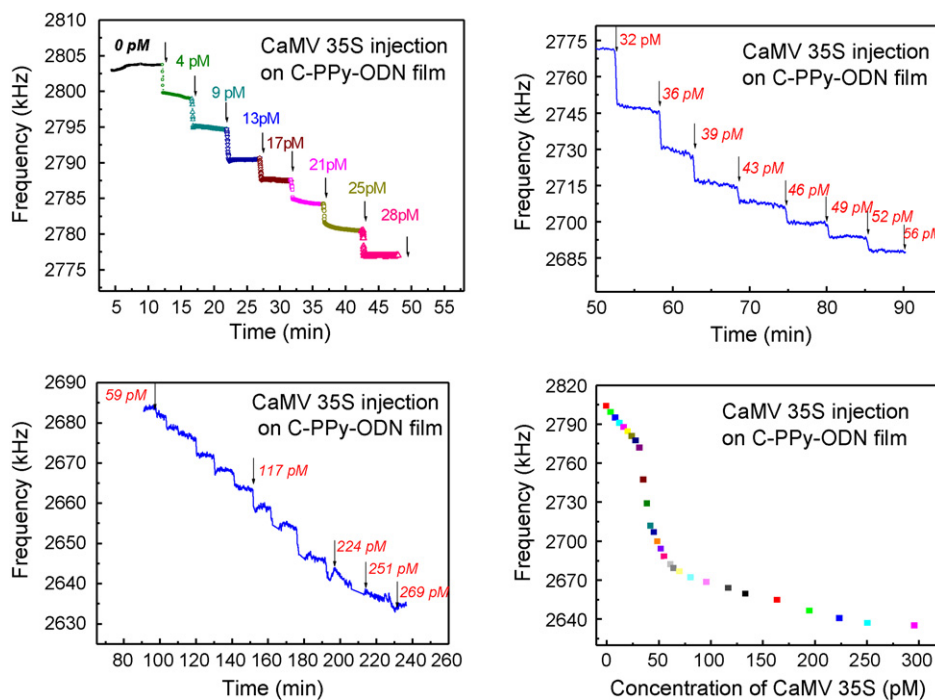


Fig. 5. QCM graph of frequency vs. time during CaMV 35S (complementary target) injection on C-PPy-ODN electrode surface. ODN target concentration varies from 1 up to 300 pM.

extremely sensitive mass variation because of its resonance frequency decrease upon the increase of a given mass on the quartz crystal. Therefore, QCM is a direct and sensitive tool to assess the immobilization of biomolecules and to evaluate their affinity to react with complementary ones in certain specific biochemical

reactions [22]. Fig. 5 shows the resonance frequency variation of a quartz electrode modified with C-PPy-ODN during the hybridization with CaMV 35S complementary strand. It can be seen from these graphs that ODN hybridization did occur at as low as 4 pM of target concentration, corresponding of mass variation of ca.

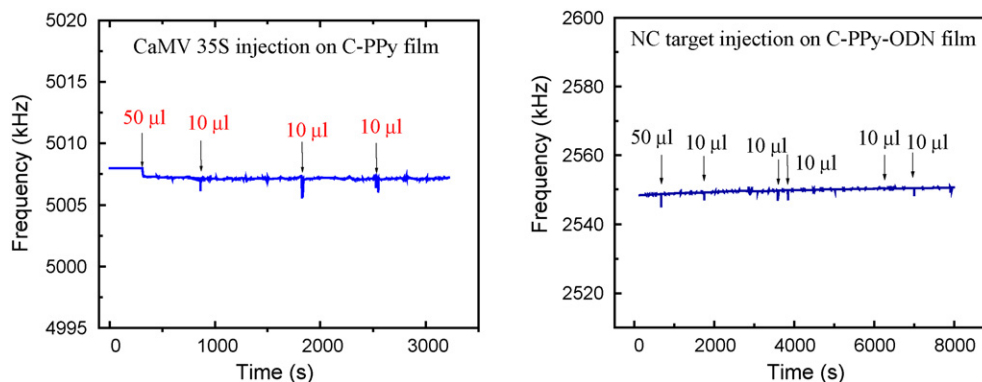


Fig. 6. QCM graph of frequency vs. time during CaMV 35S injection on ODN probe-free C-PPy film (left) and NC target (non-complementary) injection on C-PPy-ODN electrode surface (right) (blank experiments).

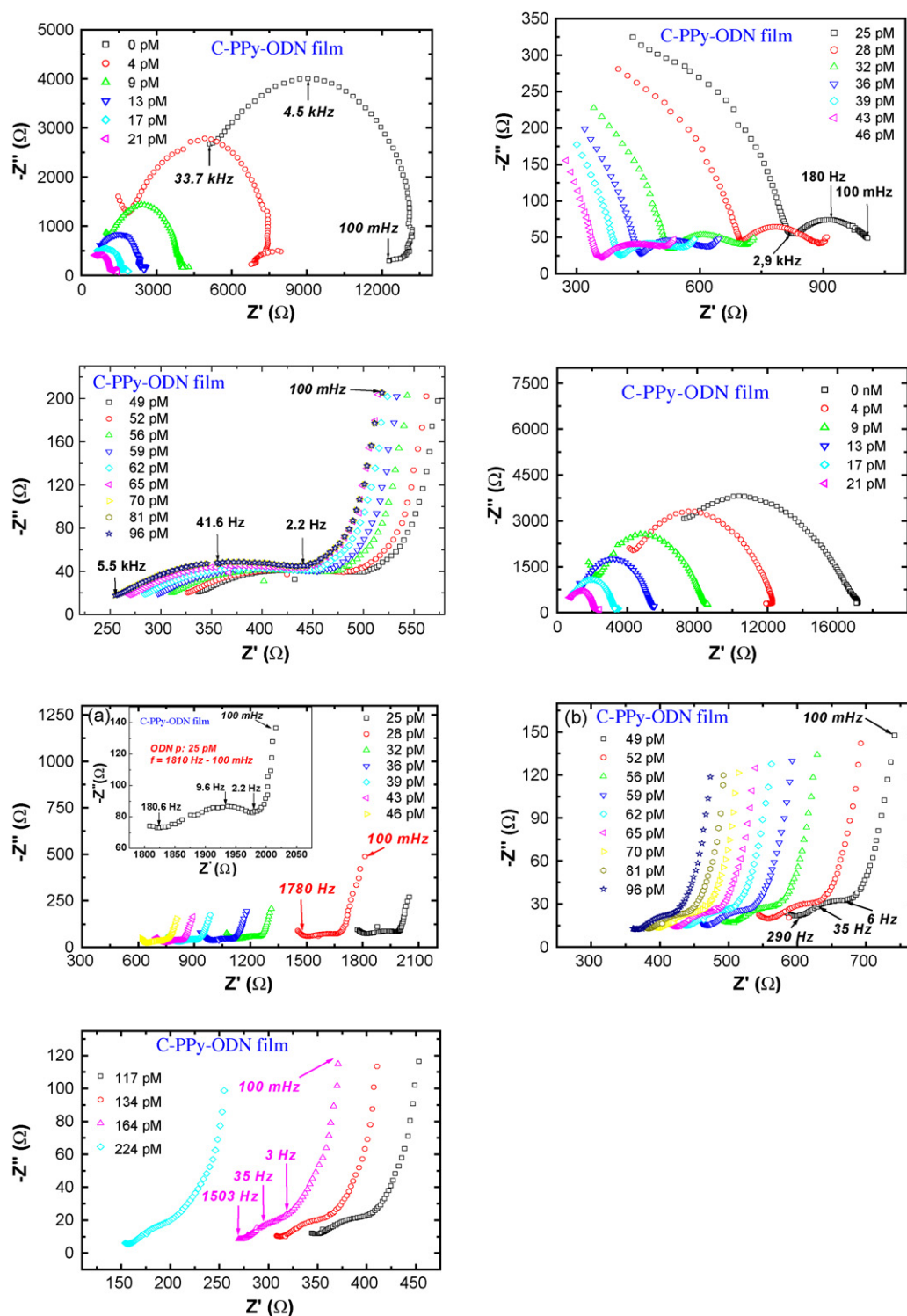


Fig. 7. EIS spectra of C-PPy-ODN films during hybridization with CaMV 35S sequence. Frequency range: 200 kHz to 100 mHz, $E_{AC} = 5$ mV, $E_{DC} = 0.32$ V vs. Ag/AgCl.

$100 \mu\text{g cm}^{-2}$. The frequency continued to fall with increasing injection volume of target. The saturation can be reached at concentration of 300 pM of target. In order to validate the transduction effect the first blank experiment with the injection of two-base mutative non-complementary (NC) target on C-PPy-ODN film and the other control of injection of complementary CaMV 35S on C-PPy film (without ODN probe!) have been carried out. In these two control experiments, the injection did not induce any frequency variation but only a slight destabilization of the quartz. It clearly

demonstrated that the resonance frequency variation in case of CaMV 35S sequence injection on C-PPy-ODN film was uniquely due to hybridization (Fig. 6).

3.3. Direct detection of CaMV 35S complementary target by EIS measurements

EIS spectra were obtained at open circuit potential. For each set of experiment (each electrode) three spectra were recorded

Table 3

Kinetic parameters, fitted with Randles model.

CaMV 35S concentration (pM)	R_s^a ($\Omega \text{ cm}^2$)	R_{ct} ($\Omega \text{ cm}^2$)	CPE ($\mu\text{F cm}^{-2}$)	N	χ^2 ($\times 10^{-4}$)
0	500	2525.0	0.2	0.85	2.6
25	500	1717.5	0.35	0.88	3.5
28	500	767.5	0.76	0.95	2.6
32	500	457.5	0.63	0.94	3.2
36	500	347.5	0.5	0.85	2.2
39	500	119.5	2.4	0.66	2.0
42	500	95.7	3.14	0.65	3.1
46	500	71.2	3.00	0.61	2.6
49	500	71.2	1.10	0.54	2.8
52	500	70.0	1.64	0.51	2.4
56	500	67.0	1.54	0.49	3.6
59	500	61.2	1.53	0.47	3.2
62	500	60.0	1.43	0.44	2.8
65	500	69.5	0.82	0.48	4.1
70	500	75.0	0.56	0.54	4.6
81	500	75.0	0.60	0.34	5.6

^a R_s was fixed at $500 \Omega \text{ cm}^2$.

corresponding to pre-ODN probe immobilization, the post-ODN probe immobilization and post-hybridization with ODN targets events, respectively. EIS Nyquist plot spectra were shown in Fig. 7. Simulated values of kinetic parameters were derived from EIS experimental data by fitting an equivalent circuit model based on Randles model [23]. This equivalent circuit includes a solution resistance (R_s); a constant phase element (CPE); and the charge transfer resistance (R_{ct}). The CPE reflects inhomogeneities of the surface layer and the substitution of the double layer capacitance with a CPE improved the adequateness of fitting. The parameter n determines the extent of the deviation from the Randles model. When $n = 1$, 0 and 0.5 the CPE represents an ideal capacitor, pure resistor and Warburg element, respectively. The values of the fitted parameters were given in Table 3. Small values of χ^2 in the range of low CaMV 35S target concentration (from 25 to 80 pM) suggest that this Randles model fit the experimental data quite well in this low range of concentration. Furthermore, the value of $1/R_{ct}$ is linearly proportional to the target concentration according to the following equation: $1/R_{ct} (\Omega^{-1}) = 0.32 \times 10^{-4} \times C - 0.3 \times 10^{-3}$ (pM). In the higher (than 81 pM) concentration data the fitting with Randles model encountered big error, related to the diffusion element of Redox probe (n approaches 0.5). For this reason Vorotyntsev's model [24] was considered to be used for a high range of concentration (96–296 pM) (Fig. 8). Previously, this model was successfully applied for quinone-containing conducting polymers [25] Vorotyntsev's model choice for our C-PPy-ODN system was rationalized on the similar “signal-on” behavior (i.e. decrease of R_{ct} during hybridization, firstly reported by Pham's group [26,27]) of this C-PPy-ODN system to the quinone one. “Signal-on” effect means that DNA hybridization at the vicinity of the polymer/solution interface increases the switching rate of electronically conducting polymer. According to the studies of this group, two main explanations can be envisaged: the electrostatic effect

and/or the steric effect due to the polyelectrolyte character of ODN strands. Both could modify the ionic transport to and across the polymer/solution interface [26–28]. However, the variation tendency of obtained kinetic parameters for C-PPy-ODN system was not fully interpreted and it will be subjected for the further study and discussion.

4. Conclusions

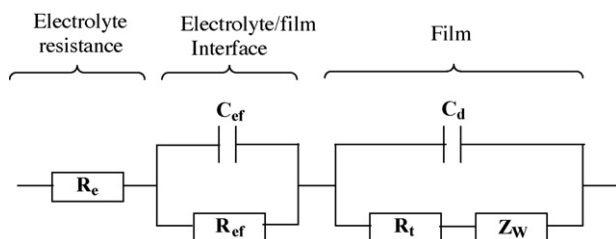
Electrochemical DNA detection is advantageous because of its fast performance and simple instrumentation. In this study, the free-label DNA detection (applied for GMO) is demonstrated based on C-PPy-ODN system. A quite low detection limit can be reached thanks to enhanced performance of C-PPy-ODN composite material. EIS data were well fitted with Randles model (in the low range of DNA concentration). In the higher range of DNA concentration, the other model, taking into account the diffusion of redox couple is needed to simulate the experimental data. Furthermore, complementary studies are necessary to interpreting all EIS values as well as understanding the signal-on mechanism of C-PPy-ODN system.

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**Fig. 8.** Equivalent circuit of Vorotyntsev's model.