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Label-free Detection of *Erwinia mallotivora* DNA for Papaya Dieback Disease using Electrochemical Impedance Spectroscopy Approach

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A labelless DNA impedance biosensor for the detection of *Erwinia mallotivora* bacteria, the causal agent for papaya dieback disease, is developed based on commercial gold screen-printed electrode (SPE). Three types of Dropsens gold SPEs with different working electrode dimensions and gold ink curing temperature (i.e. 250BT, 250AT and 223AT) were compared. Based on their peak separations via cyclic voltammetry, a 4-mm gold working electrode treated with low temperature curing ink SPE (250BT) was selected for DNA sensor development. Two unique genes selective towards *Erwinia mallotivora* namely flagellar biosynthesis protein (Flg) and hypothetical protein (Hyp) were selected for primer design, amplified, purified and immobilized on thiol-modified gold surface. Electrochemical impedance spectroscopy (EIS) analysis on gold SPE were recorded in redox solutions of 5 mM ferri/ferrocyanide in 0.1 M phosphate buffer, pH 7.0 solution prior and after the DNA hybridizations. Charge transfer resistance (R_{ct}) from Nyquist plots for both proteins increased accordingly with the DNA concentrations. The ΔR_{ct} value were found to increase from 19 kΩ to 43 kΩ for Flg and 28 kΩ to 51 kΩ for Hyp concentrations of 125 ng and 250 ng respectively.

Keywords: *Erwinia mallotivora*; DNA probe; electrochemical impedance spectroscopy; biosensor; plant disease management

I. INTRODUCTION

Papaya fruit is of considerable economic importance in Malaysia. The highly nutritious fruit has wide markets both domestically and internationally, generating millions dollar of revenues in agriculture sector. However in early 2000s, a massive papaya dieback disease outbreak has attacked our papaya industry badly causing rapid decline in production and affecting popular Malaysian export varieties like Eksotika, Solo and Sekaki. The first case reported was in 2003 by the Johor State Department of Agriculture; however by the end of 2006 the disease had spread to five other states affecting ca. 800 ha, destruction of ca. 1 million trees with total yield losses were estimated at 200 000 metric tonnes worth US$58 million (Maktar *et al.*, 2008). Consequently, Malaysia's reputation as the world's major papaya exporter had slipped from the second to fourth place with tremendous decline of more than 60% fruit production (Chan & Baharuddin, 2008; Evans & Ballen, 2012). The causal agent of this disease has been identified and confirmed as *Erwinia mallotivora* (*E. mallotivora*) (Mat Amin *et al.*, 2010); its mode of infection is by invading and colonizing the entire parts of papaya plants. Once infected, the disease is rampant and easily spread to other papaya plants especially during wet season.

Various studies have been carried out to understand the dieback plant pathogen interaction mechanism and as to halt the disease progression. Using advanced biotechnology tools, efforts also have been devoted in gene manipulation in order to produce papaya which is more resistant towards dieback disease. Recent green approach include applying 'good bacteria' spray in controlling the disease. Malaysia

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papaya industry is still reviving from the disease hence requires holistic approaches to revitalize it. An early warning system is an integral part of the holistic approach and is necessary in implementing plant disease management strategies. Such advanced detection is of paramount importance in controlling the spread of the disease hence minimizing the damage and yield/economic losses due to the pathogen infections.

The detection and identification of plant diseases could be performed via both direct and indirect methods. Laboratory-based techniques such as polymerase chain reaction (PCR), immunofluorescence (IF), fluorescence in-situ hybridization (FISH), enzyme-linked immunosorbent assay (ELISA), flow cytometry (FCM) and gas chromatography-mass spectrometry (GC-MS) are some examples of the direct detection methods (Fang & Ramasamy, 2015). Indirect methods include thermography, fluorescence imaging and hyperspectral techniques. Application of ELISA in plant disease however, has poor sensitivity for bacteria detection. On the other hands, although GC-MS, FISH, IF and FCM techniques provide excellent sensitivity, they are laboratory-based techniques with complex sample preparations/extractions which require skilled personnel to operate and professional data analyzer. The instrumentation cost for both GC-MS and FCM also is a major disadvantage for on-site detection.

For dieback disease detection particularly, a group of Australian scientist has detected the presence of the disease by observing the CO_2 exchange rate of mature leaves and carbohydrate levels in infected leaf tissue (Guthrie *et al.*, 2001). Among all direct detection methods, PCR is reported to be able to detect plant pathogen with high sensitivity and is the most common technique employed for papaya dieback detection. Albeit so, PCR has limitations such as lack of operational robustness and its effectiveness is subjected to the DNA extraction, inhibitors, polymerase activity and even concentration of PCR buffer.

Electrochemical DNA biosensor, on the other hand, is a quantification method which is more sensitive and straight-forward compared to PCR and gel electrophoresis method. This technique has been acknowledged for its robustness, sensitivity and stability. The simplicity of this system enables the fabrication of portable and user-friendly devices for on-site detection (Ronkainen *et al.*, 2010). In most DNA sensors, the target DNA must be labelled with fluorophore, magnetic beads, methylene blue or an enzyme to amplify the detection. Current trending method using electrochemically impedance spectroscopy (EIS) has great advantages in probing detection at molecular level where it requires no label and thus offer direct detection for the target analyte (Lisdat & Schäfer, 2008). The interfacial impedance, which is obtained upon application of a small AC voltage overlaid on a DC bias potential to the sensing electrode has been frequently employed for sensing various analytes (Park & Park, 2009).

Although DNA-based sensor has been widely studied in health disease and detections of GMOs, bacteria, fungi and virus; but it has yet to be utilised for plant disease detection. EIS technique in monitoring plant health reported up-to-date merely analyzing physical changes such as characterization of electrical properties of plant tissue relating to the fruits and their quality, maturation process and influence of temperature (Zwiazek & Blake, 1991; Borges *et al.*, 2012) however its application using biological elements such as DNA against its bacteria pathogen has not been fully explored.

Recently, a group of researcher from Malaysian Agricultural Research and Development Institute (MARDI) have successfully sequenced *E. mallotivora* genome (Redzuan *et al.,* 2014) and through proteomics and transcriptomics studies have identified several potential genes as candidates for early detection of the pathogen (Norliza *et al.*, 2017). By utilizing the discovered genes selective towards *E. mallotivora*, we are able to develop a DNA-based biosensor for the detection of papaya dieback disease via impedimetric approach. This finding is envisaged to be a platform knowledge in establishing advance early papaya dieback disease detection prior the appearance of any visual symptoms so that correct measures can be taken to prevent the spread of disease and countermeasures to overcome it.

II. MATERIALS AND METHODS

A. Materials and reagents

All chemicals were from Sigma-Aldrich otherwise stated. Phosphate buffered saline solutions (pH 7.0, 0.01 M) were prepared by dissolving a tablet of PBS in 200 mL of deionized water. All solutions were prepared with deionized water from Sartorius Arium® Pro Water Purification System (Sartorius, Germany). All glassware, buffers and deionized water were autoclaved beforehand. *E. mallotivora* DNA was extracted

using bacteria DNA extraction kit from Sigma-Aldrich following the manufacturer's instruction.

For electrochemical studies, all ceramic-based gold screen-printed electrodes (Dropsens, Spain) with integrated working, reference and counter electrodes were purchased from Metrohm Malaysia Sdn. Bhd. Each individual SPE has dimension of 3.4 x 1.0 x 0.05 cm and different size of gold working electrode (Table 1). A 2-mm Au probe tip, Ag/AgCl reference electrode and Pt counter electrode were also from Metrohm Malaysia Sdn. Bhd.

Table 1. Description of gold SPEs used in cyclic voltammetry analysis

Type	Working electrode dimension/Gold ink treatment	Reference electrode	Counter electrode
250BT	4-mm gold/low temperature curing ink	Silver	Platinum
250AT	4-mm gold/high temperature curing ink	Silver	Platinum
223AT	1.6-mm gold/high temperature curing ink	Silver	Gold

All oligonucleotides for sensor studies were purchased from First BASE Laboratories Sdn. Bhd. (Malaysia). DNA fragments were purified by using solid phase reversible immobilization (SPRI) beads and concentrations were checked using nanodrop (The NanoDrop® ND-1000 UV-Vis Spectrophotometer, Thermo Fisher Scientific, US). Sequences of all nucleotides are listed in Table 2.

Table 2. Sequences of DNA oligonucleotides used in the study

Description	Oligonucleotide detonation	Sequence
Hypothetical protein	HypoF1 HypoR1	TACACAGCAC(C3)ATGGCAGTGCAAGTCAGGAA CGGCGTTTTATCGCCTTGTG
Flagellar biosynthesis protein	FlgNF2 FlgNR2	TACACAGCAC(C3)CTCTCAGGCGGCCAGATAAA AGCCCGTTATGCAGATTGGT
Capture probe	Capture probe	GTGCTGTGTATTTTT-3ThiolModifierC3S-S

B. Electrochemical measurements

Unmodified gold SPEs were first characterized electrochemically via cyclic voltammetry (CV) in 1 mM ferrocenecarboxylic acid (FCA) in 0.01 M PBS pH 7.4. Following surface functionalization and oligo DNA attachment, the SPEs were analyzed using electrochemical impedance spectroscopy (EIS) in 5 mM ferri/ferrocyanide (1:1) in 0.1 M phosphate buffer, pH 7.0. Both CV and EIS measurements were performed using an Autolab PGSTAT 20 (Eco Chemie, Netherlands) with Frequency Response Analysis software for EIS. Electrochemical experiments for SPEs were carried out by dropping a 150 µL of redox solutions on the SPE surface (Figure 1) while conventional 3-electrode system with Au probe tip, Ag/AgCl reference electrode and Pt counter electrode were immersed in 10 mL of redox solution.

Figure 1. Electrochemical analysis on gold SPE is performed by dropping a 150 µL of redox solution covering all electrodes surface

C. Gold surface functionalization

Prior surface modification, gold SPEs were rinsed with 20% ethanol followed by copious amount of distilled water. A mixture of 10 mM tris(2-carboxyethyl) phosphine hydrochloride (TCEP) and 100 µM capture drop DNA was first prepared and left 2 hours at room temperature. Following the capture probe reduction, a 15 µL from 5 µM TCEP-capture probe mixtures were then dropped on the SPE, covering only the working electrode (WE) area. After 2 hours, the SPEs were washed with PBS and air-dried under nitrogen (N_2) flow. The SPEs were incubated in 1 mM 6-mercapto-1-hexanol (MCH) overnight at room temperature to reduce non-specific DNA binding. Finally, the SPEs were rinsed with PBS, air-dried with N_2 flow and 15 µL of DNA oligos with 125 ng and 250 ng concentrations were added on the WEs for 1 hour at RT before performing the electrochemical analysis.

III. RESULTS AND DISCUSSION

A. Selection of gold SPE

Electrochemical performance for 250BT, 250AT and 223AT electrodes were evaluated via cyclic voltammetry based on their peak separations (ΔE_p) and linear reduction/oxidation current (i_{pa}, i_{pc}) versus square root of scan rates (Figure 2). Gold SPE of 250BT was found to have the smallest peak separation (0.064 V), followed by 223AT (0.081 V) and 250AT (0.082 V), indicating faster electron transfer (data not shown).

The value obtained by gold SPE 250BT (0.064 V) is close to ΔE_p=0.057 mV as reported for Fc^+ ion (Elgrishi *et al.*, 2017). Furthermore, this peak separation value is comparable and as good as performance of the conventional 2-mm Au tip probe with ΔE=0.065 V thus 250BT was selected for subsequent studies.

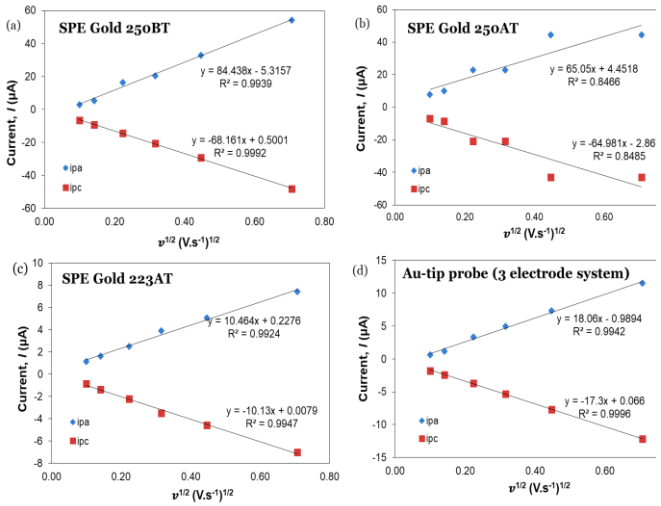

Figure 2. Current peak height of anodic and cathodic peak of cyclic voltammograms of different gold electrodes versus the square root of the scan rate in 1 mM ferrocenecarboxylic acid in 0.01 M PBS pH 7.4

B. Selection of oligo DNA

Genome comparison analysis has been carried out in six spesies of Erwinia i.e. *E. mallotivora*, *E. amylovora*, *E. billingiae*, *E. pyrifoliae*, *E. tasmaniensis* and Pebtobacterium using OrthoVenn software. There was 45 gene clusters found to be unique belonging to *E. mallotivora* (Figure 3) which contained 114 genes from the genome sequence. After further nucleotide alignment analysis; two genes were then selected for electrochemical studies; flagellar biosynthesis protein (Flg) and hypothetical protein (Hyp) as both genes contained high regions of nucleotide sequences that are unique and did not overlapped with any known bacteria sequences in the database. Two sets of primers from each sequence were further synthesized and used to generate PCR fragments using *E. mallotivora* DNA as the template. The best primer sets which gave a single clear distinctive bands and did not generate any false positive amplifications after being tested with two other known closely related Erwinia species and also papaya DNA were further utilized for the impedimetric DNA experiments.

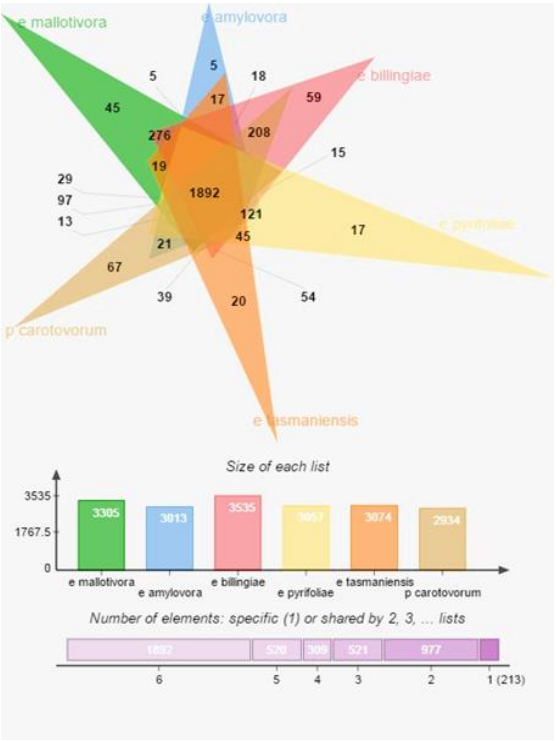

Figure 3. Erwinia genome comparison using Ortho Venn software

C. DNA sensor based on self-assembled monolayers

The DNA sensor electrodes were prepared in a two-step procedure entailing capture probe reduction and self-assembly monolayer (SAM) for surface blocking using 6-mercapto-1-hexanol (MCH). Disulfides bond at the capture probe were first selectively reduced by TCEP in water. Then the thiol-modified DNA was immobilized on a freshly clean gold SPE via chemisorption. According to Park & Park (2009), immobilization of thiolated probe DNA on the gold surface with an optimum density is a key technique for improving DNA sensing efficiencies.

In the second preparation step, MCH was bound to the DNA-modified electrode surface to prevent non-specific binding during the DNA detection experiments. Although there are also a two-step method in which probe DNA is first immobilized on the surface followed by the formation of the MCH SAM widely reported (Herne & Tarlov, 1997; Li *et al.*, 2007; Tang *et al.*, 2009), Keighley *et al.* (2008) suggested that the possibility of obtaining an optimum density of immobilized probe DNAs on the gold surface can be achieved by simultaneously immobilizing both thiolated probe DNA and MCH.

Following the MCH SAM blocking and oligo DNA attachments, each DNA-modified gold SPEs were characterized via EIS. As the surface modification is negatively charged (Kafka *et al.*, 2008), a ferri/ferrocyanide redox system in phosphate buffer solution was used for the electrochemical study.

D. Impedimetric DNA detection

A simplified schematic diagram for EIS analysis for an electrode/electrolyte interface is illustrated in Figure 4. When a DC potential is applied, solvated counter ions form an electrical double layer along the electrode surface. This double layer is represented by the double layer capacitor with a capacitance of C_{dl}. The electron transfer to/from the electroactive species, which may approach the electrode as close as two solvent molecules away in the outer Helmholtz plane (OHP), takes place across the inner Helmholtz plane (IHP) by overcoming the activation barrier, R_p (polarization resistance) and the solution resistance, R_s. Once the electron transfer gets started, the Warburg impedance (W) due to the mass transport begins to play a role in determining the electrode kinetics. In the non-faradaic EIS detection such in the case of label-free DNA sensor without redox labelling, the capacitance or the dielectric constant of the probe layer can be utilized as a main sensing signal. The charge-transfer resistance (R_{ct}), which is the polarization resistance at an equilibrium potential, is utilized as a main indicator in the faradaic EIS detection. Simply put, R_{ct} values indicate how crowded the electrode surface is when it is modified by a functional molecule. The R_{ct} values are determined by how selective binding has taken place with the analyte and how much analyte is in the test solution.

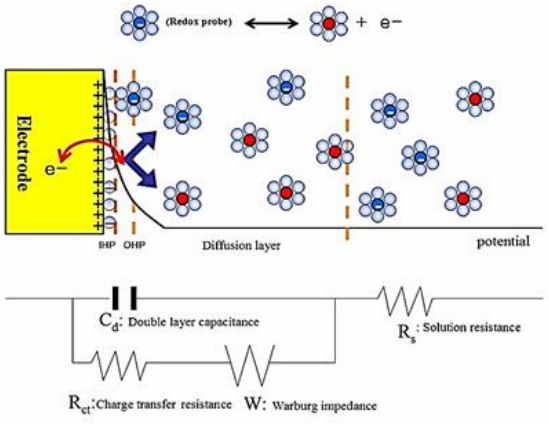

Figure 4. Schematic diagram for an electrode/electrolyte interface in a faradaic sensor with its exemplary model circuit (Adapted from Park & Yoo, 2003)

Unmodified gold SPE of 250BT in this study was found to have R_{ct} value of 517 ± 156 Ω (n=5) with f_{max} at 158.49 Hz. A typical impedance spectrum represented by Nyquist plot for the unmodified gold surface in redox solution exhibit a semi-circle shape followed by a straight line at low frequency (Figure 5a). In general, the semi-circle region at high frequency (left region on the Nyquist plot) attributed to charge-transfer process involving non-Faradaic process. The straight line emerged at lower frequency (right region on the Nyquist plot) on the other hand, correlates with diffusion mass-charge transfer associated with Faradaic reaction.

Impedance spectra of a sensor electrode after gold surface functionalization and oligo DNA attachments of 125 and 250

ng concentrations is shown in Figure 5b. The rate of charge transfer from the modified SPE to a redox indicator of $[Fe(CN)_6]^{3-/4-}$ measured by EIS in the form of charge transfer resistance (R_{ct}), is modulated by the concentration of oligo DNA selectively hybridized on the surface. EIS curve for modified SPEs with attached DNA appeared to be flattened semi-circles with no diffusion as indicated by the absence of straight at low frequency region. The increase of resistance after surface modifications with DNA is clearly visible with the increasing semi-circles shape.

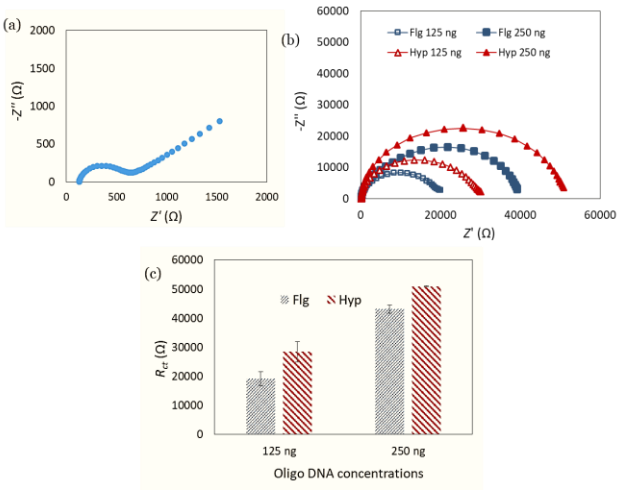

Figure 5. Nyquist plots for (a) unmodified gold SPE; and (b) gold SPE after attachment with flagellar biosynthesis proteins (Flg) and hypothetical protein (Hyp) of different concentrations in 5 mM $[Fe(CN)_6]^{3-/4-}$ in 0.1 M phosphate buffer, pH 7.0 (Frequency 0.1 Hz-100 kHz, applied amplitude 0.05 V); (c) Charge transfer resistance, R_{ct}, calculated from Nyquist plots' semi-circles for 125 and 250 ng of proteins

The R_{ct} values for both Flg and Hyp proteins increased linearly when the concentrations were doubled from 125 ng to 250 mg (Figure 5c). R_{ct} for Flg were found to increase from 19,148 ± 2,451 kΩ to 43,146 ± 1,361 kΩ. For Hyp proteins, R_{ct} values of 28,444 ± 3,479 kΩ and 50,985 ± 119 kΩ were measured for 125 ng 250 ng concentrations respectively. The changes in resistances or capacitances of the interface are induced by the DNA hybridization events with target DNA on a suitably designed probe platform (Table 3).

In terms of electrical equivalent circuit, the changes in Nyquist plots from Figure 5a to Figure 5b can be explained as follows. Impedance of a macroelectrode generally can be represented with a Randles equivalent circuits (Figure 6a). This circuit consists of R_s (solution resistance); C_{dl} (double-layer capacitance); R_{ct} (charge transfer resistance) and Z_d (diffusion impedance). In case of macroelectrode associated with plane 1-D diffusion, Z_d is represented by complex Warburg impedance:

$$Z_d = \frac{W}{\sqrt{j\omega}} = \frac{W}{\sqrt{\omega}} - j\frac{W}{\sqrt{\omega}} \quad (1)$$

where W is Warburg coefficient (or Warburg constant). At high frequencies where $R_{ct} >> |Z_d|$, diffusion impedance can be neglected. Thus, the equivalent circuit is reduced to the circuit presented in Figure 6b. The impedance spectrum of this circuit is a semicircle with the radius equal to R_{ct} and the frequency where the imaginary part of impedance takes its maximum values equal to $f_{max}=1/(2\pi C_{dl}R_{ct})$ (Hleli *et al.*, 2006). The semicircle starts from R_s and ends at R_s+R_{ct} on the real axis. The double-layer capacitance (C_{dl}) for the electrode surface can be determined from the angular frequency value (ω_{max}) based on Equation 2 and the calculated values are presented as in Table 3.

$$\omega_{max} = 2\pi f_{max} = \frac{1}{R_{ct}C_{dl}} \quad (2)$$

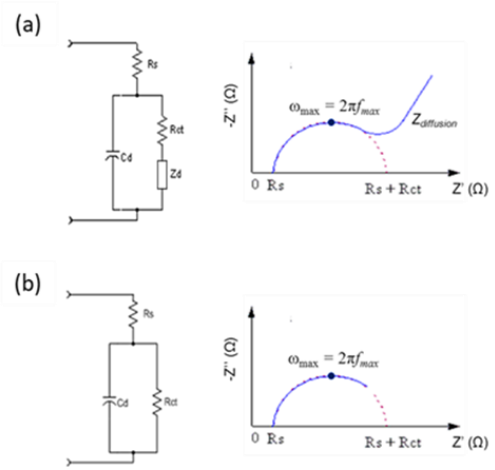

Figure 6. (a) A complete Randles equivalent circuit; (b) A reduced Randles equivalent circuit at the high frequencies, with their corresponding Nyquist plots (right inset)

Table 3. EIS parameters value for gold SPE (250BT) before and after oligo DNA attachments

Steps	R_s (Ω)	R_{ct} (Ω)	f_{max} (Hz)	C_{dl} (μF)
1.Unmodified gold SPE	114 ± 19.5	517 ± 156	158.49	1.94
2. Flg protein 125 ng	132 ± 2.05	19,148 ± 2,451	3.1623	2.63
3. Flg protein 250 ng	135 ± 2.74	43,146 ± 1,361	1.9953	1.85
4. Hyp protein 125 ng	134 ± 1.3	28,444 ± 3,479	6.3096	0.89
5. Hyp protein 250 ng	134 ± 1.3	50,985 ± 119	2.5119	1.24

Capacitive transducers' operation relies on the decrease in capacitance caused by thickening of the electrode/solution dielectric layer as a result of displacement of water and electrolyte molecules due to the immobilization and further hybridization events (Teles & Fonseca, 2008). The electrical resistance and capacitance of a surface are sensitive indicators for changes of the surface properties and the detection of DNA. Both properties are accessible by EIS. The capacitance value changes observed in this study were in the vicinity of 35-55% and were found higher than reported by Kafka & co-workers (2008) for their DNA hybridization study.

However, due to the inconsistency of the f_{max} recorded for C_{dl} calculation, the relative resistance change was chosen as sensor parameter for the DNA detection.

IV. CONCLUSION

We have shown that impedimetric detection of non-labelled DNA at different concentrations is feasible. The sensor surface was initially modified with a thiol-modified electrode. Additional blocking with 6-mercapto-1-hexanol (MCH) was necessary to increase functionality of the immobilised probe DNA and to reduce unspecific DNA binding. The R_{ct} values changes accordingly from unmodified surface to 125 ng and 250 ng concentrations of oligo DNA in the vicinity of kiloΩ. In general, the changes in impedances are linearly related to the surface coverage by a target analyte in a low concentration region.

V. ACKNOWLEDGEMENT

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