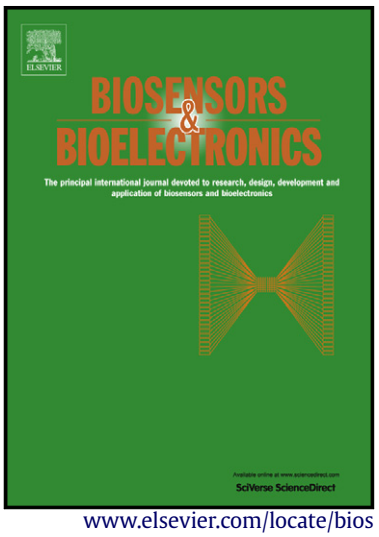

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Impedimetric DNA–biosensor for the study of anticancer action of mitomycin C: comparison between acid and electroreductive activation

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Abstract

An electrochemical protocol is described for direct monitoring of anti-cancer properties of MMC. Using electrochemical impedance spectroscopy, a pretreated pencil graphite electrode (PGE) modified with multiwall carbon nanotubes (MWCNTs) and poly(diallyldimethylmmonium chloride, PDDA) decorated with ds-DNA was employed in this study to identify DNA damages induced by MMC. The change in charge transfer resistance after incubation of the DNA biosensor in MMC solution for a known time was used as indication of DNA damage. It was found that MMC did not interact with DNA. As MMC does not inherently possess any anti-cancer activity, it is, therefore, necessary to activate it by either of two ways: activation in acidic media or electrochemical activation. Incubation of DNA–modified electrode in activated MMC led to alterations in DNA and changes in its electrochemical properties (which forms the theme of the present study). Acid and electroreductive MMC activations were compared and different adducts were subsequently generated, suggesting that the drug can bind to DNA in more than one way. Impedance spectroscopy was used for the first time as a novel technique for detecting DNA-drug adducts.

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Keywords: Mitomycin C; Electrochemical impedance spectroscopy; DNA–based biosensor; Activated Mitomycin C.

1. Introduction

Recent years have witnessed a growing interest in DNA–based biosensors and DNA interactions. Investigations in this area herald important developments in the technology, detection, and quantification of anti-cancer and carcinogen drugs and their mechanisms of action (Ensafi et al., 2013a; Sirajuddin and Badshah, 2013; Erdem and Congur, 2013; Yunus et al., 2013). The study of the interactions between drugs and DNA is a relatively little known area which forms an attractive field which is essential for gaining a deeper understanding of the mechanisms of interaction and developing efficient methods for detecting these mechanisms. The knowledge thus gained will be used for designing new, efficient drugs and for their in vitro screening (Hajian et al., 2009). The antibiotic Mitomycin C (MMC), (Scheme S–1A), is an antitumor agent used in clinical chemotherapy toward a widespread spectrum of solid tumors (United States Pharmacopeia-National Formulary, 2002; Bolenz et al., 2006). This agent was isolated in 1985 from the fermentation broth of strepturyces caespitosus (Frank, 1960). In 1974, the Food and Drug Agency (FDA) approved its administration for treating gastric and pancreatic carcinomas in combination with other anticancer agents, and it continues to be used nowadays in the chemotherapy of head and neck, cervical, gastric, pancreatic, and colon cancers (Bradner, 2001). Research in the biological mode of action of MMC was pioneered by Lyer and Szybalski in the early 1960s when they reported the two features of MMC that inspired most of the research performed in the following years: the requirement of a reductive activation to exert its biological effects and the ability of the activated drug to form DNA cross-links by covalently binding the two complementary strands of ds-

DNA (Iyer and Szybalski, 1963; Iyer and Szybalski, 1964). MMC contains two covered alkylating functions, namely C–1 aziridine and C–10 carbamate, which are located in such a way that the substance in its native form exhibits a peculiar lack of reactivity against nucleophilic attacks (Warner and Brietzke, 2008). Reduction of MMC reactivates these 'masked' functional groups, which become thoroughly alkylating and may be displaced by one or two nucleophilic centers of guanine residues in DNA (Tabaee et al., 2007).

In this study, a DNA based biosensor was constructed using the layer-by-layer technique. First, poly(diallyldimethyl-ammonium chloride), PDDA, was used as a dispersant of multiwall carbon nanotubes (MWCNTs). The MWCNTs–PDDA thus produced was then immobilized on the surface of an electrochemically pretreated pencil graphite electrode (PGE) to increase the electron transfer characteristics of the electrode surface. Finally, the ds-DNA polyanions were immobilized at the surface of MWCNTs–PDDA/PGE. The DNA–modified PGE was used as an electrochemical sensor to evaluate the anti-cancer property of the activated MMC.

The anti-cancer activity of MMC depends on both pH and potential; hence, electrochemical methods and pH alterations are used in this study to activate this property. For this purpose, electrochemical impedance spectroscopy (EIS) is used to investigate the effect of MMC on ds-DNA which is a macromolecule with negatively charged phosphate groups in its backbone. The quantity of effective negative charge of ds-DNA reduces due to the connection between activated MMC and ds-DNA. This diminishing negative charge facilitates the conversion of the ferri/ferrocyanide redox couple with negative charges which, in turn, decreases the value of charge transfer resistance (Galova et al., 2008; Ensafi et al., 2013). For the purposes of the present study, appropriate electrode materials were designed that could not only provide a foundation for fabricating electrochemical biosensors for use in the study of the anti-cancer property of MMC but also offer an in

vitro model for simulating the pathway of this property. Finally, the interactions of surface-confined DNA were compared with either acid or electroreductively activated MC.

2. Materials and methods

2.1. Chemicals

All solutions were prepared using reagent grade chemicals. Tris–HCl buffer (pH 7.0) contained 10.0 mmol L^{-1} Tris–HCl, 10.0 mmol L^{-1} NaCl and 1.0 mmol L^{-1} EDTA. Doubly distilled water was used through the work.

Phosphate buffer solution (PBS) with pH 7.0 and 4.0 (sodium dihydrogen phosphate and disodium monohydrogen phosphate plus sodium hydroxide, 0.10 mol L^{-1}) was used.

Acetate buffer (pH 4.8) contained 50.0 mmol L^{-1} acetic acid, 50.0 mmol L^{-1} sodium acetate and 2.0 mmol L^{-1} NaCl was used as a buffer medium. Mitomycin C (MMC) was purchased from Merck.

Salmon sperm ds–DNA in this work was purchased from Sigma (catalog no. D1626). The molecular weight (MW) and sequence is not determined by Sigma. The G–C% content for DNA from salmon testes is reported to be 41.2%. A salmon sperm ds-DNA stock solution (1000 mg L^{-1}) was prepared in Tris buffer (pH 7.0) and kept in frozen form. More diluted solutions were prepared with the acetate buffer containing 2.0 mmol L^{-1} NaCl.

2.2. Apparatus

Electrochemical measurements were performed using an Autolab PGSTAT 12, potentiostat/galvanostat connected to a three-electrode cell, Metrohm, Model 663 VA stand, with a GPES 4.9 software package (Eco Chemie, The Netherlands). The raw data

was treated using the Savitzky and Golay filter (level 2) of the GPES software, followed by the GPES software moving average baseline correction with a "peak width" of 0.01. The reference electrode was Ag/AgCl (3 mol L^{-1} KCl) and the counter electrode was a platinum wire. A standard one-compartment three–electrode cell of 10 mL capacity was used in all experiments. The renewable PGE, described in the study of Ozsoz and co-workers (Erdem et al., 2003), was used in all experiments. A Noki pencil was used as a holder for Pentel graphite leads. The pencil lead was a HB type spare lead (60 × 0.7 mm, Pentel; Japan). Electrical contact with the lead was obtained by soldering a metallic wire to the metallic part. The pencil was hold vertically with 12 mm of the lead being extruded outside (9 mm of which was immersed in the solution). The pencil leads were used as received. All electroanalytical measurements were performed at room temperature. Metrohm pH–meter (Model 827) with a glass electrode (Corning) was used to adjust the pH of solution.

2.3. Functionalization and purification of MWCNTs

MWCNTs from Aldrich with diameters ranging between 70 and 110 nm were employed. In this work, nitric acid supplied by Aldrich was used for the purification and oxidation of MWCNTs according to the procedure of Chicharro and coworkers (Chicharro et al., 2002). A mass of 120 mg of MWCNTs was stirred in 10 mL of a 3 mol L^{-1} nitric acid solution for 20 h. The solid product was collected on a filter paper and washed several times with pure water until the filtrate solution was neutral (pH 7.0). The functionalized MWCNTs obtained were then dried in an oven at 80° C for 24 h. Nitric acid usually causes significant destruction in carbon nanotubes and introduces –COOH groups at the ends or at the sidewall defects of the nanotube structure (Datsyuk et al., 2008). The presence of oxygen-containing groups facilitates the exfoliation of MWCNT bundles and

123 increases their solubility in polar media (Liu et al., 1998). This, in turn, affects the
124 processing of MWCNTs and increases the possibility of further
125 modification/functionalization depending on the application (Balasubramanian and
126 Burghard, 2005).

127 The MWCNTs were quantitatively analyzed by titration to determine the COOH
128 concentrations on the surface of the treated MWCNTs. In a typical experiment (Datsyuk et
129 al., 2008; Shieh et al., 2007), the carboxylated MWCNTs were added into a 25 mL 0.04
130 mol L^{-1} NaOH solution and stirred for 48 h to allow the solid MWCNTs material to
131 equilibrate with the NaOH solution. The mixture was titrated with a 0.04 mol L^{-1} HCl
132 solution to determine the excess NaOH in the solution and the concentration of the
133 carboxylates on the CNTs. After the treatment, the concentration of the acidic oxygen-
134 containing surface groups obtained was 3.4 mmol per gram of MWCNTs.

135

136 *2.4. Preparation of the DNA–based biosensor*

137 In this study, PDDA was used as a dispersant of MWCNTs. PDDA is a water soluble,
138 quaternary ammonium, and cationic polyelectrolyte that usually acts as a positively
139 charged colloid when dissolved in aqueous solutions (Li et al., 2008). The pH of the
140 double distilled water used in this study for dissolving PDDA was less than 7.0. Positively
141 charged PDDA was easily coated on the negatively charged surface of the MWCNTs by
142 electrostatic interaction. The PDDA molecules can combine considerably well with DNA
143 to form DNA films because it is a strong linear cationic polyelectrolyte (Li et al., 2008).
144 The modified electrode was fabricated according to the procedure in our previous work
145 described below.

146 Before immobilization of ds-DNA/PDDA–MWCNTs on the surface of PGE,
147 pretreatment of the surface was carried out. For this purpose, the surface of the PGE was

pretreated at +1.40 V for 300 s in a quiescent solution of (10 mL) of 0.5 mol L^{-1} of acetate buffer containing 0.02 mol L^{-1} NaCl (pH 4.8). An aqueous solution of 1.0 mg mL^{-1} PDDA was initially prepared with 0.5 mol L^{-1} NaCl. Then, 1.0 mg MWCNTs was dispersed into 1.0 mL PDDA solution. The mixture was sonicated for 3 h to obtain a homogeneous black suspension, which was sonicated for 15 min immediately before preparing the PDDA–MWCNTs films. PGEs were dipped in these composites for 60 min. The electrodes were then dipped in deionized water. The PDDA–MWCNTs/PGE was dried in N_2 stream. The positively charged PDDA–MWCNTs/PGE was subsequently dipped into the DNA solution (1.0 mg mL^{-1}, pH 7.0, buffered with Tris–HCl) for approximately 30 min, with water rinsing and N_2 drying. The modified PGEs thus obtained were designated as ds-DNA/PDDA–MWCNTs/PGE in this study. The above mentioned procedure was repeated two times to obtain the desired [ds-DNA/PDDA–MWCNTs]$_2$/PGE according to the optimized results reported in our previous work (Ensafi et al., 2013b).

2.5. Electrochemical impedance spectroscopy

EIS Measurements were carried out in the presence of 5.0 mmol L^{-1} $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ as a redox probe in 0.1 mol L^{-1} KCl (0.1 mol L^{-1} PBS (pH 4.0)) at a polarization potential of 0.10 V in the frequency range of 0.005 to 10^5 Hz and at an amplitude of 10 mV. All the experimental data were fitted using randles equivalent circuit (inserted in the EIS spectra). Due to the deviation from an ideal capacitor and a diffusion behavior, capacitance (C) was replaced with a constant phase element (CPE).

2.6. Procedure

In order to pretreat the modified electrode, [ds-DNA/PDDA–MWCNTs]$_2$/PGE was dipped into 0.1 mol L^{-1} PBS (at pH 7.0 or 4.0) for 5 min before recording the

electrochemical impedance spectra. In order to produce MMC acid activation, 0.1 mol L^{-1} PBS at pH 4.0 was used. Since complete acid activation does not occur instantaneously, MMC was dissolved in the buffer solution 24 h prior to its use in experiments. Controlled-potential electrolysis of MMC was carried out at the DNA–modified PGE in a single-compartment cell at a preselected pH (0.1 mol L^{-1} PBS). A nitrogen stream was maintained in the stirred solution during the electrolysis process. The reduction process was carried out over a given period. Electrolysis times longer than 10 min led to the precipitation of the products. The electrode was then washed twice with water and the EIS signal of the DNA–modified electrode was obtained. Measuring the differences between the charge transfer resistances (R_{ct}) of the modified electrode before and after interaction of immobilized ds-DNA with MMC was used as an analytical signal. The schematic procedure is shown in Scheme 1.

To investigate the effect of acid-activated MMC on immobilized ds-DNA, [ds-DNA/PDDA–MWCNTs]$_2$/PGE was immersed in a stirred (200 rpm) solution of the activated MMC for a short period of time (from 5 to 15 min). Then, the modified electrode was washed and the electrochemical impedance spectra were recorded. The differences between the charge transfer resistance (R_{ct}) of the modified electrode before and after interaction of immobilized ds-DNA with acid-activated MMC was used as an analytical signal.

3. Results and discussion

3.1. Surface characterization

The surface topographies of the stepwise fabrication of the DNA–biosensor were studied using SEM (Fig. 1). Figure 1A displays the SEM image of the bare PGE. The graphite layers can be clearly seen in this Figure. The MWCNTs were well dispersed in

198 the PDDA solution (Fig. 1B). When DNA was immobilized on the surface of MWCNTs–
199 PDDA, the surface morphology was changed (Fig. 1C). When the diameter of the
200 nanotubes was slightly increased, the already clear images of MWCNTs became dim.
201 Coated and uncoated surfaces of the PGE and the interface between the PGE and the
202 solution can be clearly seen in Fig. 1C. **"Here Fig. 1"**

203 Previous studies have revealed that the assembly of nucleic acids on the support may be
204 detected and traced by electrochemical impedance spectroscopy (Ensafi, et al., 2013b,
205 Ensafi, et al., 2013c). Figure 2A shows a Nyquist plot of EIS for PGE electrodes (Figure
206 2A, a), modified with PDDA–MWCNTs nanocomposite (Figure 2A, b), ds-DNA/PDDA–
207 MWCNTs (Figure 2A, c), $(\text{ds-DNA/PDDA–MWCNTs})_2$ (Figure 2A, d), and $(\text{ds-DNA/PDDA–MWCNTs})_3$ (Figure 2A, e) in 5.0 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$ (1:1) containing
209 0.10 mol L^{-1} KCl. In the Nyquist plot of the impedance spectra, the semicircle portion at
210 higher frequencies corresponds to the electron-transfer-limited process and the linear
211 portion seen at lower frequencies may be ascribed to the diffusion. The increased diameter
212 of the semicircle reflects the increasing interfacial charge-transfer resistance (R_{ct}). It was
213 found that the R_{ct} of the electrode drastically decreased in the presence of the MWCNTs–
214 PDDA nanocomposite. It may be the nanocomposite of MWCNTs and PDDA that
215 promoted the electron exchange between $[Fe(CN)_6]^{3-/4-}$ and the electrode. The increase in
216 the R_{ct} is due to the immobilization of negatively charged ds-DNA on the electrode
217 surface resulting in a negatively charged interface that electrostatically repels the
218 negatively charged redox probe $[Fe(CN)_6]^{3-/4-}$ and inhibits interfacial charge-transfer. The
219 diameter of the semicircle part increased significantly for n = 2 and then increased only
220 slightly as the bilayer number was larger than 2. With increasing n, the surface area and
221 active centers of the ds-DNA increased whereas for values of n larger than 2, the thicker
222 layers of the assembly film would cause a constant resistance to electron transfer. Thus, n

223 = 2 was selected for constructing the {MWCNTs/PDDA/ds-DNA}$_n$ film. This indicates
224 that (MWCNTs–PDDA/dsDNA)$_n$ films were successfully assembled layer-by-layer on the
225 surface of PGE with n = 2. Therefore, n = 2 was also selected for the preparation of the ds-
226 DNA–modified PGE in all the subsequent experiments.

227 **"Here Fig. 2"**

228 *3.2. Effect of MMC on ds-DNA*

229 We have previously shown that EIS is able to detect catecholics mediated ds-DNA
230 damages with high sensitivity and selectivity compared to the existing methods (Ensafi et
231 al., 2014). By oxidation of catechol in the presence of oxygen and heavy metals, hydroxyl
232 radicals ($OH^{\bullet}$) form and immediately attack ds-DNA on the biosensor to damage DNA.
233 This phenomenon alters the impedance properties of the ds-DNA–modified electrode.
234 Unlike catecholics, MMC does not produce any radicals, rather it forms DNA adducts
235 (Tomasz, 1995; Bargonetti et al., 2010).

236 In order to inspect the effect of MMC on immobilized ds-DNA, [ds-DNA/PDDA–
237 MWCNTs]$_2$/PGE was immersed in a stirred (at 200 rpm) 0.1 mol L^{-1} PBS (pH 7.0)
238 containing a known quantity of MMC for a short period of time (from 5 to 15 min). Then,
239 the modified electrode was washed and the electrochemical impedance spectra were
240 recorded. The obtained results (Fig. 2B) show that the value of R_{ct} did not change and
241 therefore MMC had no effect on the ds-DNA. In addition, this procedure was repeated
242 with a bare PGE, MWCNTs/PGE and [PDDA–MWCNTs]/PGE as a reference test. For
243 this purpose, the impedimetric response of the bare PGE, MWCNTs/PGE and [PDDA–
244 MWCNTs]/PGE were recorded before and after their immersion in a stirred (200 rpm)
245 0.1 mol L^{-1} PBS (pH 7.0) containing certain amount of MMC for short period of time
246 (from 5 to 15 min). The obtained result showed that the amount of R_{ct} had not any change
247 during the period time. This means that MMC has not any effect on the surface of these

electrodes. According to these results, we concluded that the interaction of MMC with the ds-DNA is not electrostatic because in electrostatic interaction the MMC could react with the surface of the electrode non-selectively and tends to change the amount of R_{ct}. So, we decided to test the mechanisms that enable MMC to activate for interaction with the ds-DNA. For this purpose, we focused on mechanisms that enhance the positive charge of MMC to facilitate its interaction with ds-DNA.

3.3. Activation of MMC with pH alteration

MMC has positive charges in acidic media while ds-DNA has negative charges due to its phosphate groups. Initially, investigation of MMC activation was performed in an acidic medium. Fig. 2C shows the impedimetric responses of [ds-DNA/PDDA–MWCNTs]$_2$/PGE before and after immersion in an acid activated MMC solution (1.0 μmol L^{-1}). As shown, the value of R_{ct} decreases after interaction of MMC and ds-DNA, which is good evidence that activated MMC in acidic media has the capability to interact with the ds-DNA and exhibits an anti-cancer activity. In other words, changes in the impedance of the ds-DNA modified electrode could be possibly explained by the binding of the acid-activated MMC to the ds-DNA such that MMC covalently bound to the ds-DNA immobilized at the surface is held in close vicinity of the electrode and decreases the R_{ct} of the modified electrode. Acid activation is sufficient to trigger aziridine ring opening to produce carbocation B (Scheme S-1, A) (Perez et al., 1998) which is a hard alkylating agent and reacts preferentially with the site of the highest electron density (Tomasz et al., 1993). There exists some dissension in the literature on the formation of cross-links under acidic conditions. Nevertheless, Tomasz et al. indicated that no DNA crosslinking occur under acidic conditions (Tomasz et al., 1987).

Knowing that MMC is activated in acidic media, attempts were made to observe the effect of pH on MMC–DNA interaction. In PBS with a pH level ranging between 2.0 and 7.0, 1.0 μmol L^{-1} MMC solutions were prepared one day before the measurements in order to allow for complete acid activation. Dependence of R_{ct} on the pH value of the 1.0 μmol L^{-1} MMC solution (in which the DNA–modified electrode was immersed in an open circuit for 10 min) was studied. As in previous sections above, the modified electrode was washed and the impedimetric responses (R_{ct}) were recorded. It was observed that an S–shaped curve emerged (Fig. 3A) (showing low R_{ct} at lower pH levels). Clearly, the value of R_{ct} increased with increasing pH from 4.0 to 6.0 beyond which it leveled off. Furthermore, a constant trend was observed for the R_{ct} with increasing the solution pH (in the range of 2.0 to 4.0). Thus, it is observed that interaction of MMC with ds-DNA is pH dependent. Thus, PBS (pH 4.0) was selected for activating MMC in acidic media because the value of R_{ct} was minimum at this pH level which is also closer to biological pH compared with the other two pH levels for an equal value of R_{ct}. The additional advantage of the selected pH level is that the amount of acid consumed is less than that with the other two pH levels for an equal value of R_{ct}. In other words, at pHs > 4.0, there is no possibility of MMC activation to interact with the ds-DNA; this is because no carbocations could be produced (otherwise, stronger acidic conditions would be needed). Immersion of the DNA–modified electrode in buffer solutions in the absence of MMC resulted in no changes in R_{ct} values for the pH range studied. **"Here Figure 3"**

In order to inspect the effect of MMC concentration on the immobilized ds-DNA, [ds-DNA/PDDA–MWCNTs]$_2$/PGE was incubated in a stirred (at 200 rpm) 0.1 mol L^{-1} PBS (pH 4.0) containing different concentrations of MMC (00.0 to 20.0 mmol L^{-1}) for 10 min. Then, the modified electrode was rinsed with distilled water and the electrochemical impedance spectra were recorded. As shown in Fig. 3B, the value of R_{ct} (estimated from

297 the spectra) decreased up to an MMc concentration of 10.0 μmol L^{-1} beyond which it
298 leveled off. The interaction of the ds-DNA and MMC tends to decrease the amount of
299 negative charge of ds-DNA. Decreasing the effective negative charge of the ds-DNA
300 facilitates the redox reaction of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ as a redox probe and decreasing
301 the value of R_{ct}. It may, therefore, be concluded that a very low concentration of MMC is
302 required for the interaction of MMC activated in an acidic medium with ds-DNA.
303
304 *3.4. Effect of electrochemically reduced MMC on ds-DNA*
305 As a second objective, we pursued the interactions of surface-confined ds-DNA with
306 electro-reductively activated MMC considering the fact that MMC/ds-DNA interaction
307 depends on potential (Marin et al., 1998). Prior to the study of reductive activation of
308 MMC, the voltammetric behavior of MMC at the unmodified–PGE had to be investigated.
309 The electrochemistry of MMC in different solvents had been previously reported in the
310 literature (Rao et al., 1977; Teijeiroa et al., 1995; Andrews et al., 1986). Figure 4A shows
311 the CV for the signal of 100.0 μmol L^{-1} MMC in 0.1 mol L^{-1} PBS (pH 7.0) at an
312 unmodified–PGE in the potential range between Ei = 0.00 V and E_{sw} = –0.75 V. There are
313 two cathodic peaks and two anodic peaks, which can be attributed to a series of redox
314 processes of MMC. The mechanism of the reduction of MMC at pH 7.0 is thought to
315 belong to the ECE (electrochemical-chemical-electrochemical) class, where C represents
316 one or more steps (Rao et al., 1977). The electrochemical steps are considered to be R–R
317 (reversible-reversible) (Rao et al., 1977) while the chemical steps are considered to be
318 irreversible. The initial electrochemical step responsible for the waves and peaks
319 designated as I has a reversible CV peak potential at –0.40 V for the reduction wave and at
320 –0.38 V for the oxidation wave at pH 7.0. This step is a reversible two-electron, two-
321 proton reduction of the quinone structure to the hydroquinone (Rao et al., 1977). The

chemical reaction of Mitomycin is demethoxylation and aziridine opening following the reduction to the hydroquinone. The ring opening of the reduced form is very rapid, of the order of seconds (Rao et al., 1977), compared to the ring opening of the oxidized form. The process responsible for the waves and peaks, designated by II, has a reversible CV peak potential of –0.53 V for the reduction and –0.52 V for the oxidation wave at pH 7.0. This is a two-electron, two-proton reduction of the ring-opened MMC to the corresponding hydroquinone. The pH dependence of the peak potential for I_c corresponds to a slope of 60.2 mV/pH that expected for a two-electron and two-proton process at pH > 5 (Fig. S-1), whereas at pH < 5.0, the plot of E *vs.* pH had a slope of over 92.0 mV/pH unit. For II_c the value of the peak potential *vs.* solution pH was varied with pH by a slope of 92.0 mV/pH unit for pH < 5.0, and 57.1 mV/pH unit for pH > 5.0 (Fig. S-1). Moreover, these waves are not affected by making the solution acidic (pH<4) for the period of time.

"Here Figure 4"

To investigate the influence of electrode potential on the interaction of MMC with the ds-DNA at the electrode surface, a potential range covering the electrochemical reduction of MMC was employed using constant-potential electrolysis. [ds-DNA/PDDA–MWCNTs]$_2$/PGE was immersed into a stirred PBS (pH 7.0, at 200 rpm) containing 1.0 μmol L^{-1} MMC and a negative potential (E_C) was applied to the electrode for 1 and 2 min. Then, the electrode was washed and the EIS signal of the electrode was recorded. The R_{ct} was used as a measure of the degree of the interaction at the surface-attached DNA during the electrode treatment. For this purpose, a constant potential, thin-layer electrolysis was carried out at a potential range between a potential less negative than I_c and a potential more negative than II_c. Therefore, thirteen potentials were applied from 0.00 to –0.80 V. The results obtained (Fig. 4B) showed that the value of R_{ct} remained constant up to –0.30 V before it reduced. In addition, the value of R_{ct} at –0.60 V reached a constant value. The

data obtained from the comparison of interaction times (1 and 2 min) showed no significant differences so that it may be claimed that the interaction between activated MMC and ds-DNA occurs in less than 1 min. In this case, MMC was activated at the electrode simultaneous with its binding with the ds-DNA, contrary to what occurred in the case of acid activation where activation took place in the solution prior to interaction with DNA. In the absence of DNA and with H^+ as the nucleophilic center, the main result upon total reduction of MMC is 2,7-diaminomitosene (Kumar et al., 1998), in which the aziridine ring is not present while the weaker carbamate alkylator group is intact. This product alkylates DNA monofunctionally. However, in the presence of ds-DNA, the bifunctional activation path was conspicuous when the first MMC reduction step was also fast (Tomasz, 1993) as it was in the case of MMC electro-reduction. The first alkylation step (Kumar et al., 1998) occurs with the intermediacy of quinone methOde (Scheme S-1B, compound No. 5), then the Michaele limination of C–10 carbamate was favorable, and the product thus obtained proved to be receptive to a second guanine attack (Scheme S-1B), leading to the formation of the bisadduct (cross-link) (Scheme S-1B, compound No. 8) (Tomasz, 1995).

In the final stage of this study, the effect of pH on the electrochemical reduction of MMC was inspected. For this purpose, according to the data presented in Fig. S-1, at pHs lower than 7.0, the electrochemical reduction of MMC was resulted at potentials less negative than that of pH 7.0. Therefore, for applying a constant potential in different pHs (2.0 <pH< 6.0),–0.60 V was selected. Undoubtedly, in this potential, the electrochemical reduction of MMC has been perfectly occurred in the pH range from 2.0 to 6.0. For the assessment of the pH effect and potential on the MMC-DNA interaction, [ds-DNA/PDDA–MWCNTs]$_2$/PGE was immersed into a stirred (at 200 rpm) 0.1 mol L^{-1} PBS in the pH range from 2.0 to 6.0 containing 1.0 μmol L^{-1} MMC. Then, a constant potential

of–0.60V was applied for 1 min. The modified electrodes were rinsed with water and the electrochemical impedance spectra were recorded. The results (Figure 5) showed that the value of R_{ct} reached its minimum in the pH range from 4.5 to 6.0,. As shown in Figure 5, when the DNA–modified electrode was immersed in MMC acid equilibrated solution (pH ranging between 2.0 and 4.0) at potential –0.60 V, the R_{ct} value ($R_{ct}\approx$ 4.0) was lower than the one obtained exclusively with acid activation at open circuit ($R_{ct}\approx$ 4.6) (see Figure3A) but higher than that with electroreductive activation at neutral pH ($R_{ct}\approx$ 3.2). As explained above, in acidic media, MMC coexists in solution with the activated form 2 (Scheme S-1B). At lower pH levels, the acid activated form predominates and the monofunctional adduct (compound No. 7, Scheme S-1B) is obtained. Under these conditions, the quantity of MMC in its native form (susceptible to electroreduction) is low. Therefore, we tentatively propose a mixture of monofunctional (compound No. 7, Scheme S-1B) and the cross-link 8. In the control DNA, the DNA–modified electrode was immersed for 1 min in PBSs not containing MMC; no significant changes were observed in the R_{ct} values in the studied pH range.

"Here Figure 5"

3.5. Reproducibility and stability

To test the stability of the electrochemical sensor, EIS signals were measured before and after incubation in a PBS (pH 7.0) containing 1.0 μmol L^{-1} MMC. EIS measurement was repeated within several days at the same biosensor. Between the measurements, the sensors were stored under dry conditions in the open air at room temperature. The working stability was characterized by the RSD value of the EIS signal obtained from 4 consecutive measurements where the RSD values were 6% and 7% for the acid and electroreductive activations, respectively. The total stability of the sensor was tested by measuring the marker signal every three days during a period of 21 days. The RSD values

reached 15% (n = 10) for acid activation and 18% (n = 10) for electroreductive activation. The stability and reproducibility values can satisfy the impedimetric assay of the interactions of the surface attached dsDNA with MMC.

4. Conclusion

In this work, a novel and simple in-vitro method was utilized to mimic the pathway of MMC–DNA interaction in a DNA layer-by-layer film. A uniform and stable multilayer film was constructed by successive adsorption of PDDA–MWCNTs and negatively charged natural DNA onto the PGE surface. MMC–DNA interaction was assessed using EIS as an analytical technique. Based on the results obtained, MMC itself has no interaction with DNA such that this property must be somehow activated. So, the effects of pH and MMC concentration were investigated to find that the interaction of MMC and ds-DNA reaches its maximum in acidic media due to both the positive charge of MMC and the formation of carbocations. The results obtained from investigation of pH effects on MMC activation revealed the occurrence of MMC electrochemical reduction. These studies showed that alkylation and cross-linking of ds-DNA into MMC might have occurred during MMC reduction. The results also showed that EIS could be used as an appropriate technique for investigating the action mechanism of anti-cancer drugs, especially those that need to be activated.

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Legends for the figures

Fig. 1. SEM image of **A)** bare PGE; **B)** [PDDA–MWCNT]/PGE; and **C)** [ds-DNA/PDDA–MWCNT]$_2$/PGE.

Fig. 2. A) Impedance spectra of a): bare PGE; b): MWCNTs–PDDA/PGE; c): {MWCNTs–PDDA/DNA}/PGE; and d): {MWCNTs–PDDA/DNA}$_2$/PGE e) {MWCNTs–PDDA/DNA}$_3$/PGE in 5.0 mmol L^{-1} $Fe(CN)_6^{3-/4-}$ containing 0.10 mol L^{-1} KCl; **B)** the effect of MMC on impedimetric response of [ds-DNA/PDDA–MWCNT]$_2$/PGE after incubation in a stirred (200 rpm) 0.1 mol L^{-1} PBS (pH 7.0) containing certain amount of MMC for short period of time (from 5 to 15 min); **C)** Electrochemical impedance spectra of [ds-DNA/PDDA–MWCNT]$_2$/PGE before a) and after 5 b), 10 c) and 15 min d) immersion in activated MMC solution (1.0 μmol L^{-1}).

Fig. 3. A) The effect of activation pH on R_{ct} of [ds-DNA/PDDA–MWCNT]$_2$/PGE after immersion in 0.1 mol L^{-1} PBS with different pH containing 1.0 μmol L^{-1} MMC for 10 min; **B)** The effect of MMC concentration activated in acidic media on R_{ct} of [ds-DNA/PDDA–MWCNT]$_2$/PGE after immersion in 0.1 mol L^{-1} PBS (pH 4.0) containing different amount of MMC (00.0 to 20.0 mmol L^{-1}) for 10 min.

Fig. 4. A) Cyclic voltammogram of 100.0 μmol L^{-1} in 0.1 mol L^{-1} PBS (pH 7.0).at scan rate of 200 mV s^{-1}; **B)** The effect of activation potential on R_{ct} of [ds-DNA/PDDA–MWCNT]$_2$/PGE after immersion in 0.1 mol L^{-1} PBS (pH 7.0) containing 10.0 μmol L^{-1} MMC.

511 **Fig. 5.** The effect of pH of MMC solution on R_{ct} of [ds-DNA/PDDA–MWCNT]$_2$/PGE
512 after immersion in 0.1 mol L^{-1} PBS (pH 7.0) containing 10.0 μmol L^{-1} MMC
513 (condition: applying the constant potential –0.6 V for 1 min).

514 **Scheme 1.** The schematic diagram of work.

515

➔ An electrochemical protocol for direct monitoring of MMC-DNA interaction is described.

➔ Pencil graphite electrode-modified with MWCNTs decorated with ds-DNA was used to identify the MMC-DNA interaction.

➔ Electrochemical impedance spectroscopy as a novel technique is used to monitor DNA-drug adducts.

➔Incubation of DNA-modified electrode in activated MMC leads to alter the EIS response.

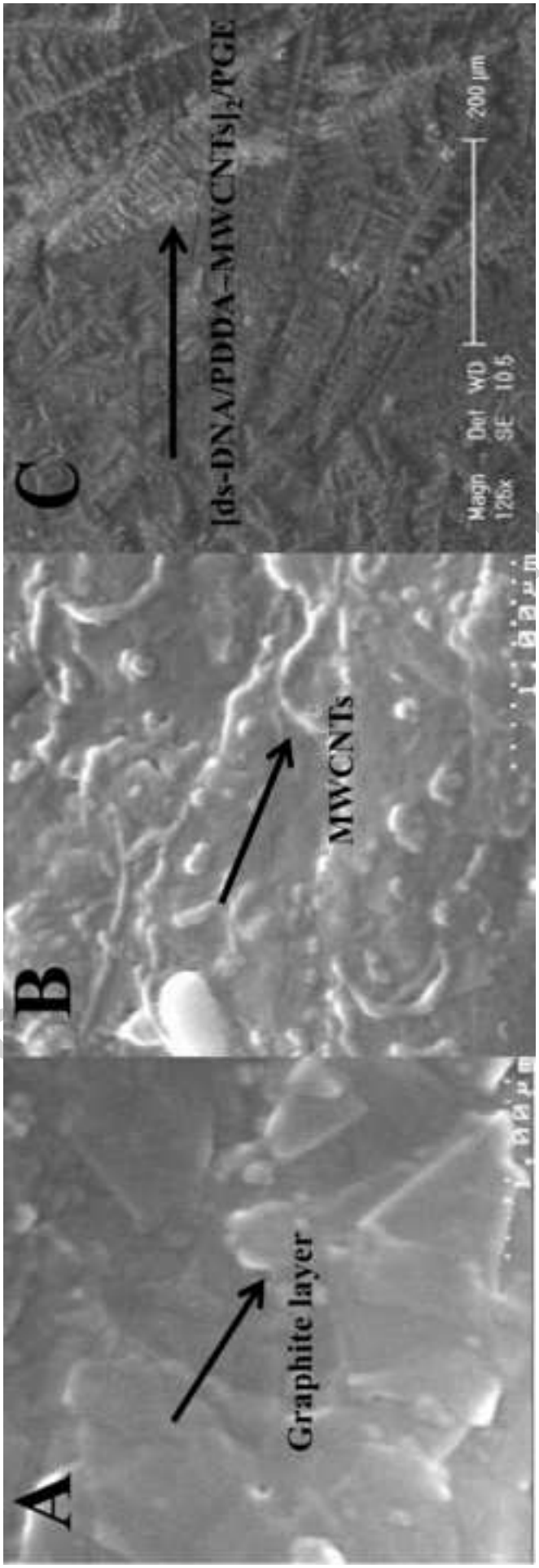


Figure 1

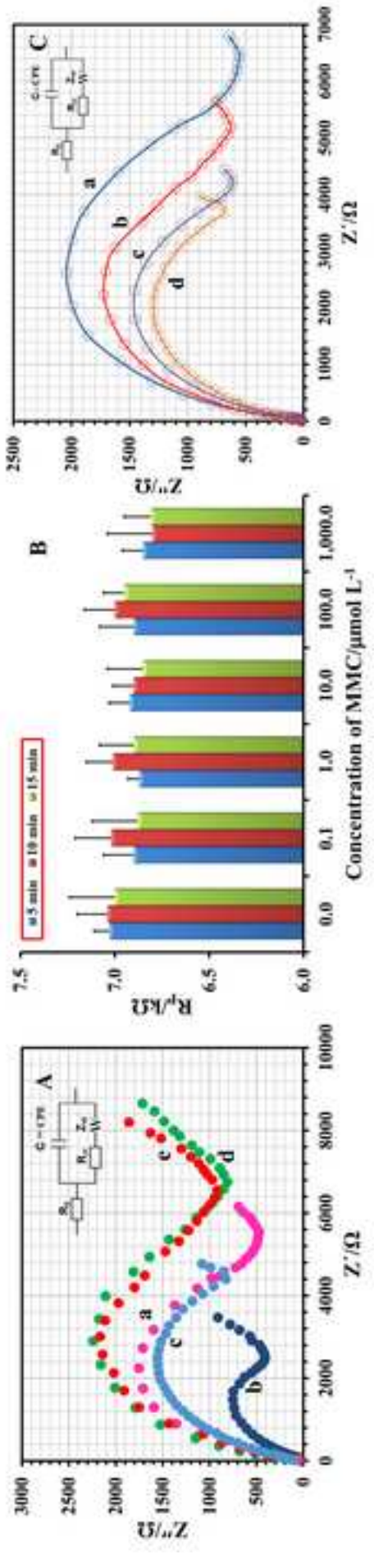
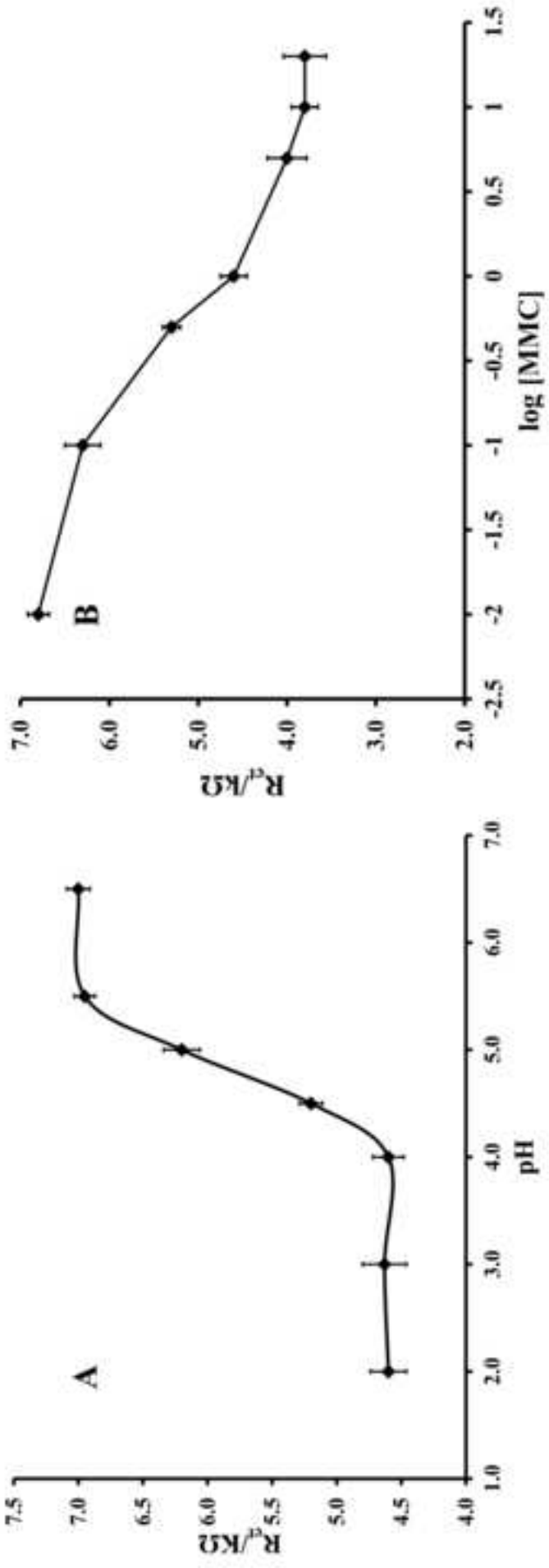


Figure 2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B
$R_{cl}/k\Omega$
pH
log [MMC]
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7.0
6.5
6.0
5.5
5.0
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4.0
1.0
2.0
3.0
4.0
5.0
6.0
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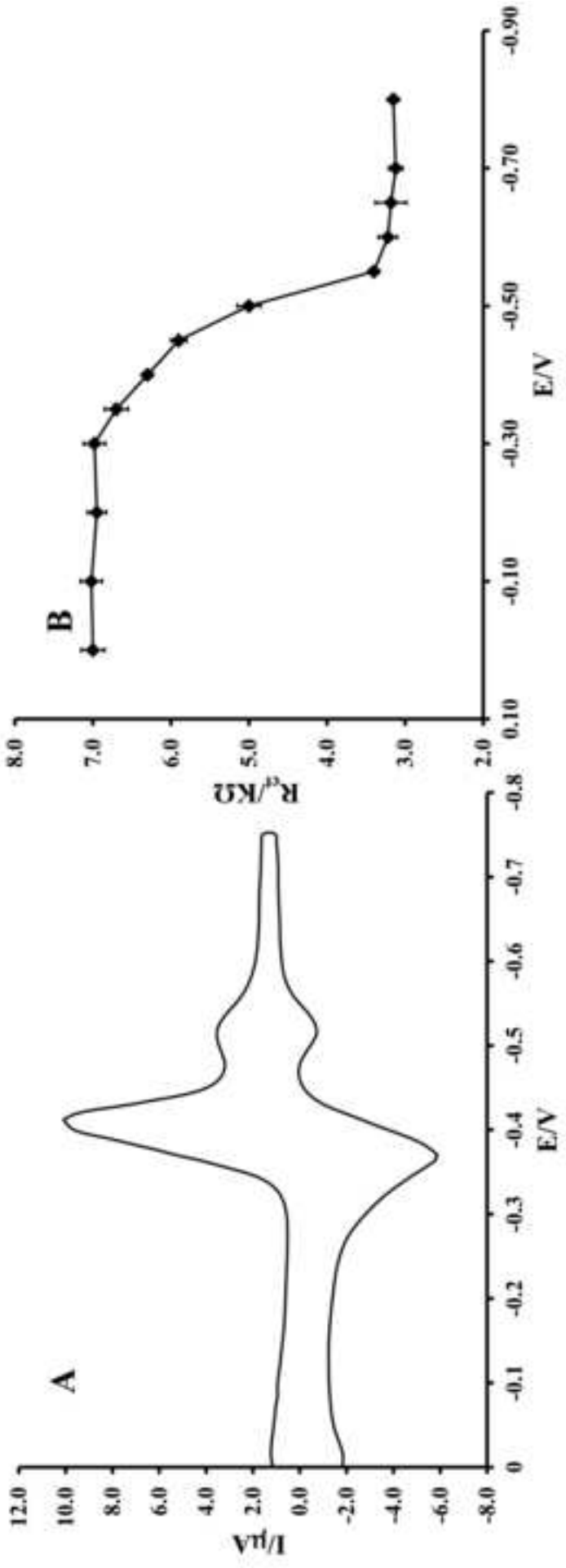
-1
-0.5
0
0.5
1
1.5

Figure 3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I/μA
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8.0
6.0
4.0
2.0
0.0
-2.0
-4.0
-6.0
-8.0
0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
-0.8
E/V
B
R_{ct}/KΩ
8.0
7.0
6.0
5.0
4.0
3.0
2.0
0.10
-0.10
-0.30
-0.50
-0.70
-0.90
E/V

Figure 4

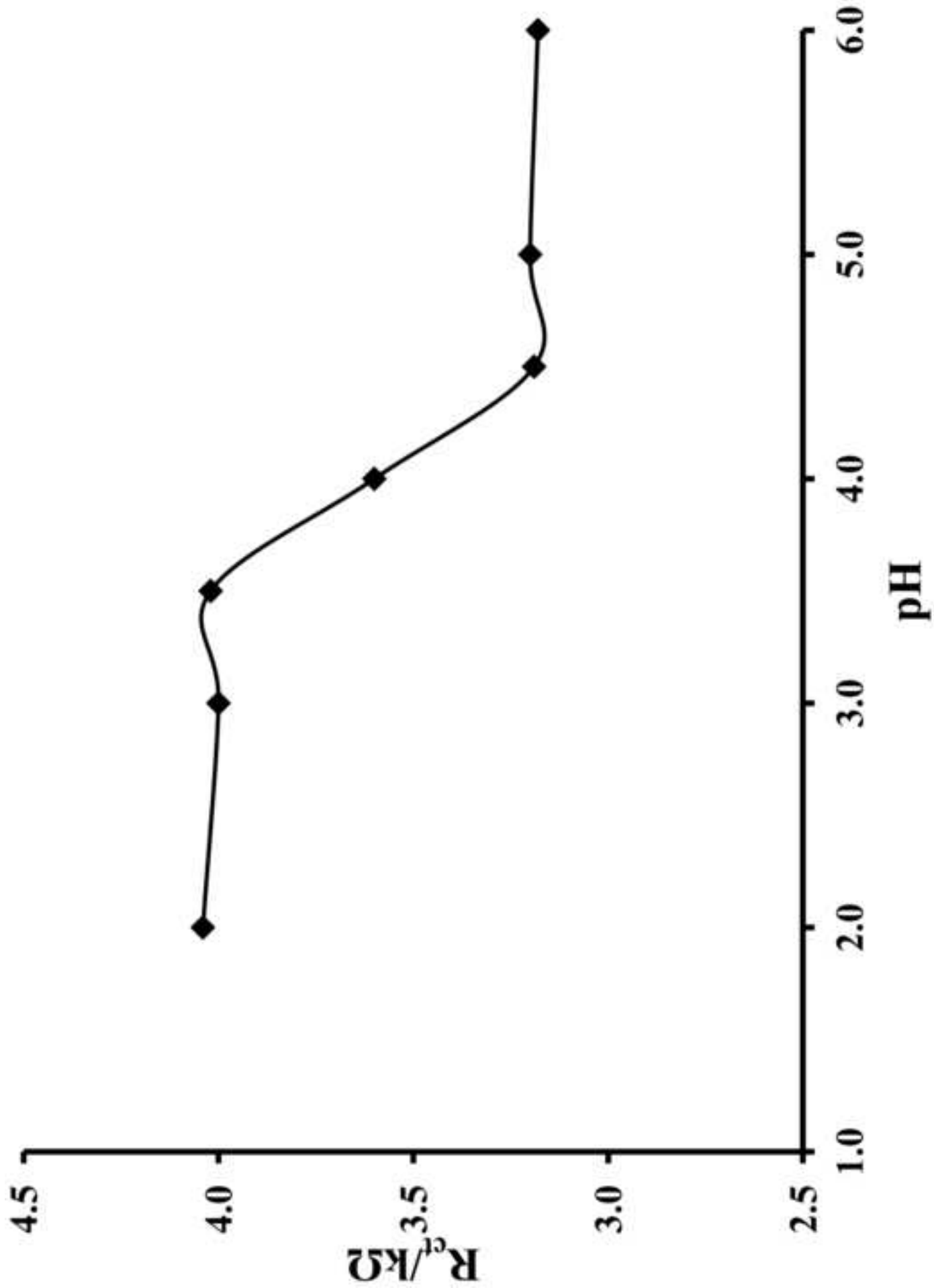

Figure 5

Scheme 1