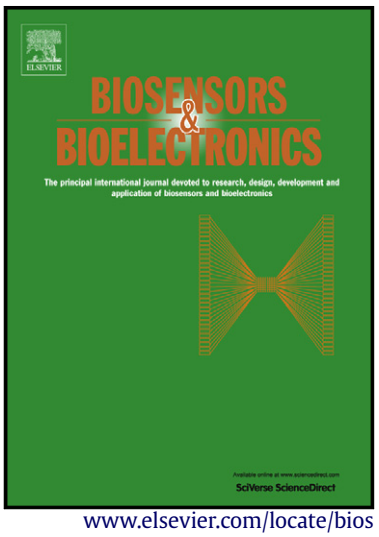

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Assessment of genotoxicity of catecholics using impedimetric DNA–biosensor

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Abstract

The potential toxicity of catecholics is a big concern, because the catechol-derived semiquinone radical after oxidation of catechol (CA) can donate a H–atom to generate quinone, and during this process a superoxide anion radical may be produced. Considering the fact that catecholics are highly consumed in our daily life and some drugs also contain one or more CA moieties, we speculate that CA's toxicity might not be insurmountable. Therefore, finding approaches to investigate catecholics potential toxicity is of great significance. Here in, an electrochemical protocol for direct monitoring of genotoxicity of catecholics is described. CA encapsulated on MWCNTs (CA@MWCNT) through continuous cyclic voltammetric on the surface of pencil graphite electrode (PGE). Subsequently, a DNA functionalized biosensor (DNA/CA@MWCNT/PGE) was prepared and characterized for detection and investigation of DNA damage induced by radicals generated from catecholics. The change in the charge transfer resistance (Rct) after incubation of the DNA biosensor in the damaging solution for a certain time was used as an indicator for DNA damage. Incubation of DNA-modified electrode with CA solution containing Cu(II), Cr(VI) and Fe(III) has been shown to result in oxidative damage to the DNA and change in the electrochemical properties. It was found that presence of Cu(II),

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Cr(VI) and Fe(III) in solution caused damage to DNA. The inhibitory effect of glutathione and plumbagin on the CA-mediated DNA damage has also been investigated using the biosensor. The minimum concentration of the metals ions for CA induced DNA damage was investigated. Recognition of suitable matrixes for CA-mediated DNA damage can be assessed using proposed DNA biosensor. Such direct monitoring of the DNA damage holds great promise for designing new biosensors with modification of the biosensor with different damaging agents.

Keywords: Catechol; DNA damage; Electrochemical impedance spectroscopy; DNA based biosensor; Plumbagin; Glutathione.

1. Introduction

Induction of oxidative damage (oxidative stress) is a noticeable event to explain metal-induced mutagenicity and genotoxicity. Various carcinogenic metals such as cobalt, chromium, lead, mercury, and copper cause redox reactions in living systems. These metals can produce the reactive oxygen species (ROS) and reactive nitrogen species (RNS) in vivo and vitro systems in experimental and clinical medicine (Price and Joshi, 1983; Casalino et al., 1997; Halliwell and Gutteridge, 1999). ROS is created during several mechanisms such as: 1) generation by neutrophils and macrophages during inflammation, 2) irradiation by gamma rays, UV light and by X–rays, 3) in reactions that catalyzed by metal, 4) in mitochondria-catalyzed electron transport reactions and other mechanisms (Cadenas, 1989). The ROS/RNS are known to play important roles in biological systems and they can be either harmful or beneficial to living systems (Valko, 2004). The ROS has physiological roles in cellular responses to anoxia, as for example in protection against contagious agents and in the function of a number of cellular signaling

systems. Furthermore, ROS at low concentrations can induce a mutagenic response. In contrast, at high concentrations, ROS can be main intervener of damage to cell structures, including lipids and membranes, proteins and nucleic acids (Poli et al., 2004). The highly reactive hydroxyl radical ($OH^{\bullet}$) is one of the most important radicals that reacts with DNA by addition to double bonds of DNA bases and by abstraction of an H atom from the methyl group of thymine and each of the C–H bonds of 2-deoxyribose (Sonntag, 1987). Non-enzymatic antioxidants and antioxidant enzymes can balance the harmful effects of ROS (Halliwell, 1996). During studies in the past few decades have shown metals like iron, copper, cadmium, mercury, nickel, lead and arsenic possess the ability to produce reactive radicals, resulting in damage like depletion of enzyme activities, damage to lipid and DNA (Chen et al., 2001). These reactive radical species contain oxygen, carbon, sulfur and nitrogen radicals and are produced from two sources. First, from the hydrogen peroxide, lipid peroxides and superoxide radical, second, chelates of proteins complexes, amino-acids and peptides with the toxic metals. These metals generate reactive species that cause neurotoxicity, hepatotoxicity and nephrotoxicity in humans and animals (Halliwell, 1996; Chen et al., 2001) The generation of free radicals, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), is termed oxidative stress and induces a cellular redox imbalance, which is linked to cancer incidence (Stohs and Bagchi, 1995).

Catechol (CA) (1,2 dihydroxybenzene) is a cytotoxic, immunotoxic, and genotoxic metabolite of benzene (Baretto et al., 2009; Schweigert et al., 2001; Valko et al., 2004). The international agency for research on cancer (IARC) classified catechol as a group 2B carcinogen, which means that it is possibility carcinogenic to human (Bolton et al., 2000). This organic compound is an important industrial chemical and occurs naturally in certain foods such as anion, crude beet sugar and coffee (IARC working group, 1999). It forms

also in digestion of some drugs, notably aspirin (Oikawa et al., 2001). CAs readily undergoes auto-oxidation in an aqueous solution, at physiological pH, to form semiquinone radicals and quinones, which are more reactive than CAs. The mechanisms most frequently cited to explain the toxicity of CAs are: 1) generation of ROS by redox reactions; 2) DNA damage in the form of oxidative damage or DNA arylation; 3) protein damage by sulfhydryl arylation or oxidation and 4) interference with electron transport in energy transducing membranes (Opperhuizen, 1985). CA has been reported to induce 8-oxodG in a human leukemia cell line HL–60 (Madamanchi et al., 2005) suggesting that CA compounds reach genomic (or mitochondrial) DNA, and Cu plays a key role in this type of DNA damage. Copper is known to exist in chromosome and closely associated with DNA bases to maintain homeostasis (Agarwal et al., 1989). However, the quantitative nature of the reaction between catecholics and DNA, especially in the presence of transition metals, remains to be clarified.

To the best of our knowledge, there is no report on fabricating electrochemical biosensors in detecting CA–induced DNA damage. Therefore, designing suitable electrode materials that can provide a foundation for fabricating electrochemical biosensors in detecting CA–induced DNA damage and offer an in vitro model to simulate the pathway of DNA damage and protection in living process is highly necessary. In this work, CA as damaging agent is one of the components of the sensor. Here, CA encapsulated on MWCNTs (CA@MWCNT) through continuous cyclic voltammetry (CV) on modified pencil graphite electrode scans in the CA solution. After this a DNA functionalized biosensor (DNA/CA@MWCNT/PGE) was prepared and characterized for detection and investigation of DNA damage induced by radicals generated from catecholics. Such procedures lead to strong adsorption (immobilization) of CA as damaging agent on the carbon surfaces. We investigated the oxidative DNA damage induces by CA in presence

of metal ions with electrochemical impedance spectroscopy (EIS). Furthermore we inspected the role of glutathione and plumbagin in DNA damage prevention.

2. Materials and methods

2.1. Chemicals

All solutions were prepared using reagent grade chemicals and doubly distilled water was used through the work. Acetate buffer (pH 4.8) was used as a buffer medium, which contained 50.0 mmol L^{-1} acetic acid, 50.0 mmol L^{-1} sodium acetate and 2.0 mmol L^{-1} NaCl. 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. 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 (total concentration of phosphate was 0.10 mol L^{-1}).

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 of the ds–DNA were prepared with an acetate buffer solution (0.5 mol L^{-1}, pH 4.8) containing 0.02 mol L^{-1} NaCl. Glutathione, CA, $CoCl_2$, $CuC1_2$, $MnC1_2$, $FeC1_3$, $NiC1_2$, $ZnC1_2$, $K_2Cr_2O_7$ and $CdC1_2$ were purchased from Merck (Darmstadt, Germany). Reagent grade Tris–HCl, MWCNTs, H_3PO_4, NaCl, plumbagine and NaOH were purchased from Aldrich Chemicals.

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. 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.

Multiwall carbon nanotubes (MWCNTs) were purified and functionalized as described elsewhere (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 the 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.

2.4. Preparation of DNA based biosensor

Before immobilization of MWCNTs and ds–DNA on the surface of PGE, 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 (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 3.0 mg mL^{-1} MWCNTs in ethanol was prerared. The mixture was sonicated for 3 h to obtain a

151 homogeneous black suspension, which was sonicated for 15 min immediately before
152 preparing the MWCNTs films. PGEs were dipped in these composites for 60 min and
153 dried in room temperature. After this stage electrochemical CA encapsulation was
154 performed according to (Kumar and Swetha, 2010). For this purpose dried MWCNTs
155 modified PGEs immersed in an electrochemical cell containing 1.0 mmol L^{-1} CA in PBS
156 (pH 7.0). Electrochemical-assisted CA encapsulation was done by potential cycling
157 treatment of the MWCNT/PGE electrode between –0.40 to 1.00 V in this solution at a
158 scan rate of 50 mV s^{-1} (n = 30). Then, CA@MWCNT/PGE was immersed in 1000 mg L^{-1}
159 DNA (Tris buffer solution, pH 7.0) for 360 minutes and it dried in air. The modified PGEs
160 thus obtained were designated as DNA/CA@MWCNT/PGE in this study.

161 For the construction of DNA/MWCNT/PGE and DNA/CA@MWCNT/PGE,
162 MWCNT/PGE and CA@MWCNT/PGE were immersed in 1000 mg L^{-1} DNA (Tris buffer
163 solution, pH 7.0), respectively for 360 min and then they were dried in air.

164

165 *2.5. Electrochemical impedance spectroscopy*

166 EIS Measurements were carried out in the presence of 5.0 mmol L^{-1}
167 $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ as a redox probe in 0.1 mol L^{-1} KCl (0.1 mol L^{-1} PBS pH 4.0)
168 at a polarization potential of 0.10 V in the frequency range of 0.005 to 10^5 Hz and at
169 amplitude of 10 mV.

170

171 *2.6. Optimization of assembly film*

172 In the process of biosensor preparation several variables such as the amount of
173 MWCNTs, time of MWCNTs immobilization and CA concentration were examined and
174 optimized with electrochemical impedance spectroscopy.

In order to inspect the effect of amount of MWCNTs, different amounts of MWCNTs between 0.1 and 0.4 mg mL^{-1} were selected. The pretreated PGEs were immersed in these solutions for 1 h and dried overnight in air. Then, MWCNT/PGE was transferred to the electrochemical cell containing $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ and the impedance spectra was recorded. Obtained results (Fig. S1) showed that with increasing the amount of MWCNTs up to 0.3 mg mL^{-1} the charge transfer resistance decreased and then leveled off. Therefore, 0.3 mg mL^{-1} of the MWCNTs was selected and used in all further experiments. In order to select the best immobilization time of MWCNTs, the pretreated PGEs were immersed in 3.0 mg mL^{-1} suspension of MWCNTs for different times (between 20 to 100 min) and dried in air overnight. Then, MWCNT/PGE were transferred to the electrochemical cell containing $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ and impedance spectra was recorded. The obtained results (Fig. S2) showed that with increasing in the time until 60 min, the charge transfer resistance (R_{ct}) decreased and then leveled off. Therefore, 60 min was selected as an optimum time for the immobilization of MWCNTs on the surface of PGE.

In order to obtain the optimum concentration of CA for the encapsulation, MWCNT/PGEs were prepared according to the optimum conditions and then it transferred to the electrochemical cell containing different CA concentrations between 0.1 and 10 mmol L^{-1} in PBS (pH 7.0). Then, electrochemical-assisted CA encapsulation was carried out by potential cycling treatment of MWCNT/PGE electrode with scan rate of 50 mV s^{-1} (n = 30). In the final stage CA@MWCNT/PGEs were transferred to the electrochemical cell containing $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ and the impedance spectra were recorded. The results (Fig. S3) showed that with increasing CA concentration until 1.0 mmol L^{-1} the impedance spectra increased and then leveled off. So 1.0 mmol L^{-1} CA was selected as an optimum concentration for the encapsulation.

200 *2.7. Procedure*

201 Investigations of DNA damage induced by CA in the presence of metal ions and
202 some antioxidants and scavengers were carried out by means of EIS. To detect the DNA
203 damage induce by CA in presence of some metallic ions such as copper, the constructed
204 modified PGEs in above section was immersed in Tris buffer solution (pH 7.0) containing
205 the metallic ions. To follow the DNA damage, impedance measurements were carried out
206 for DNA/CA@MWCNT/PGE before and after immersing in the metallic ions. In the final
207 stage of our study, the effect of glutathione and plumbagin (as antioxidant) on the DNA
208 damage was investigated. For this purpose different concentrations of antioxidants was
209 added to damaging solution and the impedance spectra was recorded.

210

211 *2.8. HPLC analysis of 8-oxodG*

212 The reaction mixture contained dsDNA (0.5 mg) and CA (100.0 μmol L^{-1}) in the
213 absence and presence of the transition metals (20.0 μmol L^{-1}) in Tris buffer solution (pH
214 7.0). After keeping this solution at room temperature for a certain time, the sample was
215 centrifuged at 3000 rpm for 5 min. The supernatant was discarded and then 0.5 mL of
216 60% (v:v) formic acid was added. The sample was hydrolyzed for 45 min at 150 °C. After
217 the hydrolysis, the formic acid was removed by freeze-drying. The residues were
218 solubilized in distilled water and then were applied to HPLC for analysis of 8HOG using
219 the method of Kaur and Halliwell (1996) with a minor modification. The HPLC analysis
220 was carried out on a Nucleocil 3 μm C_{18} column (30 cm × 4.6 mm) with the eluent of 20
221 mmol L^{-1} sodium citrate, 17 mmol L^{-1} acetate buffer (pH 3.0) and methanol (9:1) at a flow
222 rate of 1ml:min. 8HOG was detected by electrochemical detector at a potential of 0.85 V.

223

224

3. Result and discussion

3.1. CA encapsulation

The electrochemical encapsulation of CA onto the MWCNT/PGE was carried out under the condition in 2.4. Fig. 1A shows the consecutive cyclic voltammetric response of the MWCNT/PGE with 1.0 mmol L^{-1} CA in PBS buffer (pH 7.0). Upon continuous cycling, two couple of redox peaks centered at 0.023 and 0.220 V (oxidation peak) *vs.* Ag/AgCl (sat'd KCl) were developed, which is indicated that the encapsulating of the CA onto the MWCNTs (Kumar and Swetha, 2010). After encapsulation process, CA@MWCNT/PGE was thoroughly washed with distilled water and dried at room temperature. **"Here Fig. 1"**

3.2. Electrochemical properties and surface characterizations

Fig. 1B shows cyclic voltammogram of a CA@MWCNT/PGE recorded in 0.1 mol L^{-1} PBS (pH 7.0). As shown, the anodic peak currents (Ipa_1 and Ipa_2) and cathodic peak currents (Ipc_1 and Ipc_2) of CA@MWCNT/PGE were directly proportional to the scan rate in the range from 10 to 100 mV s^{-1} (Fig. S4) and the ratio of anodic peak currents to cathodic peak currents (Ipa/Ipc) was almost equal to unity (Fig. 1B) for the both peaks. The peak-to-peak separation ($\Delta Ep = E_{pa} - E_{pc}$) was 650 mV and 400 mV for ΔEp_1 and ΔEp_2, respectively at low scan rate (5 – 20 mV s^{-1}). The electron transfer number and the surface coverage were calculated according to the following equations:

$$I_p = (n^2F^2\Gamma\nu A)/4RT$$

$$Q_\Gamma = nFA\Gamma$$

where n is the number of electrons, A is the working electrode area, ν scan rate (50 mV s^{-1}) and other parameters are usual meanings (Bard and Faulkner, 1980). The electron transfer number and the surface coverage were calculated to be 2.10 and 43.7×10^{-10} mol

250 cm^{-2}, respectively. The cyclic voltammograms of CA@MWCNT/PGE electrode was
251 strongly affected by the solution pH (Fig. S5). An increase in the solution pH caused a
252 negative shift in the Ep values with slopes of –61 mV pH^{-1}, which is close to the expected
253 value of –59 mV pH^{-1} for involving same number of electron and proton coupled electron
254 transfer process (Bard and Faulkner, 1980).

255 Alternative current (AC) impedance spectroscopy was used to identify the effect of
256 the modification. For this purpose, impedance spectra of a bare PGE, MWCNT/PGE and
257 CA@MWCNT/PGE were recorded. As shown in Fig. 2A the R_{ct}, the diameter of the
258 semicircle, decreases dramatically after modification of PGE by MWCNTs, which is due
259 to the acceleration of electron transfer. It was found that the impedance of the electrode
260 drastically decreased in the presence of MWCNTs. It may be due to promotion of electron
261 transfer rate between $[Fe(CN)_6]^{3-/4-}$ and the electrode surface. Furthermore, as shown in
262 Fig. 2A with encapsulation of CA inside the MWCNTs, the amount of R_{ct} was increased.
263 Encapsulated CA inside the DNA reduces conductivity of MWCNTs and therefore the R_{ct}
264 increases. **"Here Fig. 2"**

265 Figures 2B and 2C show transmission electron microscopy (TEM) image of
266 MWCNTs and CA@MWCNTs films, respectively. A noticeable amount of black spots in
267 this figure confirms that CA were encapsulate successfully onto the MWCNTs.

268 The nucleic acid bases of DNA have chemical groups (e.g., amines) that can
269 donate electrons to the oxidized nanotube surface. The immobilization principle of DNA
270 can be explained by the formation of covalent coupling between the carboxylated ends of
271 MWCNTs and the amine groups of the DNA (Erdem et al., 2006). Nakashima et al.
272 (2003) suggested that the π-π interactions between the nanotube sidewalls and the nucleic
273 acid bases may be responsible. There may also be some weak interaction between the
274 major and minor grooves of the DNA and the nanotubes. To construct the

DNA/CA@MWCNT/PGE, CA@MWCNT/PGE immersed in 1000 mg L^{-1} DNA (Tris buffer solution, pH 7.0) and dried in air. Obvious increase in the interface impedance (Fig. 2A) due to formation of the negatively charged DNA layer on the electrode surface confirms that the DNA has been absorbed at the surface of the modified electrode.

3.4. DNA damage by catechol plus metal ions

The impedimetric detection of DNA damage has been accomplished using an electroactive compound, i.e. $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$. It is suggested that the surface-attached DNA could decelerate the rate of electron transfer due to the electrostatic repulsion between the negatively charged of the DNA and $Fe(CN)_6^{3-/4-}$. This phenomenon increases the R_{ct} (Figure 2A). The damage of ds–DNA by damaging reagents decreases the charge transfer rate, by reducing the negative charge on the electrode surface. The diminished negative charge facilitates the conversion of the ferri/ferrocyanide redox couple. Therefore, the change of the surface-attached DNA after incubation in the damaging solution was indicated by a decrease in R_{ct} due to an increase in the electrochemical reversibility of the redox couple (Galova et al., 2008; Ensafi et al., 2013)

The effect of CA on the DNA layer at the electrode surface was examined after the incubation of DNA/MWCNT/PGE in a CA solution both in the presence and absence of the transition metals ions. To detect the influence of CA on DNA damage, DNA/MWCNT/PGE was incubated in CA solution (100.0 μmol L^{-1}) both in the absence and presence of the transition metals ions (20.0 μmol L^{-1}) in Tris buffer solution (pH 7.0) for a given time under stirring. Then, DNA/MWCNT/PGE was rinsed with water and the impedimetric responses was obtained in 5.0 mmol L^{-1} $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ containing 0.10 mol L^{-1} KCl in 0.1 mol L^{-1} PBS (pH 4.0). The results showed no obvious change in the Rct with CA alone, in the absence of the metal ions (Figure not shown),

indicating that CA itself is not a DNA damaging agent. Cu(II), Cr(VI) and Fe(III) induced decreasing in the Rct in the presence of CA (Fig. 3A), whereas Mn(II), Co(II), Ni(II), Zn(II) and Cd(II) showed little or no effect on the Rct under the same conditions. It may be claimed, therefore, that Cu(II), Cr(VI) and Fe(III) induced damage to DNA in the presence of CA. The metal ions alone (in the absence of CA) induced little or no significant change in the impedimetric parameters and did not induce any DNA damage (Figure not shown). When ranked in terms of their capacity to make DNA damage with CA, the following arrange was obtained: Cu(II) > Fe(III) > Cr(VI). **"Here Fig. 3"**

In order to compare the results obtained with the DNA based sensor developed, the 8-OxodG of these samples were determined by a HPLC assay (Figure 3B). After interacting $OH^{\bullet}$ with the DNA, guanine yields an oxidation product of 8-OxodG. The most common procedure employed to assay oxidative DNA damage is the measurement of 8-OxodG by HPLC with electrochemical detection (HPLC-ECD) after hydrolysis of DNA (Floyd et al., 1986; Kasai et al., 1986). This procedure has the advantage that 8-OxodG is a major product of DNA damage, generated by attack of several reactive oxygen species. As shown in Figure 3B, the HPLC results are in good agreement with the impedance spectra obtained under similar conditions.

The dependence of catechol concentration (in the range of 0.0 – 1000.0 μmol L^{-1}) on the impedimetric signal after incubation (2 h) of DNA/MWCNT/PGE in the CA solution plus Cu(II), Cr(VI) and Fe(III) (20.0 μmol L^{-1}) was studied (Figure 3C). The results showed that the value of Rct decreased with increasing CA concentration. As shown in Figure 3B, the rate of these changes is much more for Cu(II). The results also suggest that CA is rapidly autoxidized in the presence of Cu(II), causing DNA damage.

3.5. Investigation of catechol mediated DNA damage using DNA/CA@MWCNT/PGE

As a second goal of this study, we investigated the possibility of employing a CA incorporated MWCNTs (CA@MWCNT) DNA biosensor as an analytical tool to assess any catechol mediated DNA damage. In fact, construction of a biosensor that can specially follow DNA damage mediated by a particular reagent group (CA derivatives) can be helpful to investigate mechanism and parameters those influence on the damage. For this purpose, we developed DNA/CA@MWCNT/PGE as a catecholics mediated DNA damage sensor with encapsulation of CA into MWCNTs.

Fig. 4A shows the impedance spectra of DNA/CA@MWCNT/PGE before and after immersing in Tris buffer solution (pH 7.0) containing 20.0 μmol L^{-1} Cu(II), Fe(III) and Cr(VI). As shown in this figure the amount of R_{ct} was decreased after the incubation. In order to prove the role of CA and Cu(II), Fe(III) and Cr(VI) on the damage of DNA and applicability of the proposed biosensor, reference test was done. For this purpose DNA/MWCNT/PGE was fabricated without CA encapsulation and its impedance spectra was recorded after immersion in 20.0 μmol L^{-1} Cu(II), Fe(III) and Cr(VI) for 2 h under stirring (200 rmp). No significant change was observed in the R_{ct} of DNA/MWCNT/PGE before and after incubation in the metals ions solutions. Therefore, it can be concluded that Cu(II), Fe(III) and Cr(VI) alone have not potential of DNA damage. Furthermore, we repeated the reference test with DNA/CA@MWCNT/PGE. In this stage DNA/CA@MWCNT/PGE was immersed in Tris buffer pH 7.0 (free of Cu(II)) for 2 h under stirring (200 rmp) and after washing with distilled water, it was transferred into the electrochemical cell to record the impedance spectra. The obtained results showed that CA without Cu(II), Fe(III) and Cr(VI) have not potential of DNA damage. These observations give us permission to use the proposed DNA biosensor for investigation of catecholics mediated DNA damage. As mentioned before, the 8-OxodG of these samples were

determined by a HPLC assay with electrochemical detection (HPLC-ECD) after hydrolysis of DNA (Floyd et al., 1986; Kasai et al., 1986). **"Here Fig. 4"**

The interface area of DNA/CA@MWCNT/PGE before and after incubation of the electrode in a solution containing the damaging agents (the metals ions) in $K_3Fe(CN)_6$ solution was obtained from the slope of the I_{pa}–$\nu^{1/2}$ as described in supplementary material. In DNA/CA@MWCNT/PGE before incubation in the Tris buffer solution (pH 7.0) containing the damaging metals ions, the electrode surface was found as 0.2016 cm^2. For DNA/CA@MWCNT/PGE after incubation in the Tris buffer solution (pH 7.0) containing 20.0 μmol L^{-1} Cu(II), Fe(III) and Cr(VI) was 0.2831, 0.2512 and 0.2339, respectively. Increase in the interface surface area of DNA/CA@MWCNT/PGE electrode in contact with the electrolyte, suggesting increasing the porosity of the films as a result of the DNA damage (Amirudin and Thierry, 1996). This observation is in agreement with the EIS results.

In order to investigate the Cu(II), Fe(III) and Cr(VI) concentration on the catechol mediated DNA damage using the proposed DNA biosensor, DNA/CA@MWCNT/PGEs were constructed according to the optimum conditions in section 2.4 and they were immersed in different concentrations of the metals ions between 0.0 to 100.0 μmol L^{-1} in Tris buffer (pH 7.0) with stirring (200 rmp) for 2 h. Then, DNA/CA@MWCNT/PGEs washed with distilled water and transferred in the electrochemical cell containing $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ to record the impedance spectra. As shown in Fig. 4B, change in R_{ct} was observed when the concentration of Cu(II) was 0.01 μmol L^{-1}. No significant change was observed in Rct with 0.01 μmol L^{-1} Cr(VI), showing that CA, in the presence of 0.01 μmol L^{-1} of this transition metal, is not a DNA damaging agent. A notable change in Rct was observed when the concentrations of Cr(VI) was 1.0 μmol L^{-1}. This

observation confirms that the minimum concentrations of Cr(VI) for CA induced DNA damage is approximately 100 times higher than those of Fe(III) and Cu(II).

Incubation time is one of the important parameters affects on the damage. In order to investigate the effect of this parameter, fabricated DNA/CA@MWCNT/PGEs were transferred to Tris buffer (pH 7.0) solution containing 20.0 μmol L^{-1} Cu(II), Fe(III) and Cr(VI) under the stirring condition (200 rmp) between 0 and 120 min. Then, the CA@MWCNT/PGEs were washed with distilled water and they transferred to the electrochemical cell containing $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ to record the impedance spectra. As shown in Fig. 4C no significant change was observed in the Rct before 5 min with Cu(II) and Fe(III) and before 10 min for Cr(VI). Furthermore, the Rct was decreased until 75 min for Cu(II) and 90 min for Fe(III) and Cr(VI), and then leveled off for more time. It may be claimed; therefore, depending on the type of the metals ions, radical production is stopped or reduced after a definite time.

Fig. 5 compares the results of the analysis of CA induced DNA damage in different solution matrices after 2 h incubation of DNA/CA@MWCNT/PGE. The results showed that although CA itself did not cause DNA damage in deionized and doubly distilled water, it could induce DNA damage in industrial wastewater, drinking and distilled water. Ordering the inducing effects on CA–dependent DNA damage yields: industrial wastewater > drinking water > distilled water. The order was consistent with the existence of the transition metals ions in these matrices. We masked the contribution to the reaction from metals ions impurities by complexing them with an effective chelating agent. EDTA and l,l0–phenanthroline were chosen in studying the catechol oxidation. They are most effective at a high pH range, where they act as a sexadentate ligand occupying all coordination sites of the complexed metals ions. It is also important to note that the two chelating agents used are not capable of acting as an oxidation inhibitor in the

399 classic sense of terminating free-radical chains. Addition of complexing agents such as
400 EDTA or l,l0–phenanthroline, in order to mask the present metal ions, reduced the DNA
401 damage rate (Table S1). **"Here Fig. 5"**
402
403 *3.6. The effect of glutathione and plumbagin on DNA damage induce by catechol and*
404 *Cu(II)*
405 In the third set of experiments, the antigenotoxic potential of glutathione and
406 plumbagin were evaluated using electrochemical impedance spectroscopy. As shown in
407 Figure 6A, the Rct of DNA/CA@MWCNT/PGE after incubation in Tris buffer (pH 7.0)
408 containing 20.0 μmol L^{-1} Cu(II) and 20.0 μmol L^{-1} glutathione is greater than for Cu(II)
409 alone. Therefore, it can be concluding that glutathione has the capability of decreasing the
410 DNA damage or antioxidant effect in this system and proposed DNA biosensor can be
411 recognized this effect. Furthermore, the effect of plumbagine on catechol-mediated DNA
412 damage has been compared with antioxidant action of glutathione, as illustrated in Fig.
413 6A. As can be seen, both antioxidants including GSH and plumbagine diminish the DNA
414 damage by radicals in a concentration dependent manner. It is apparent that GSH is far
415 more effective than plumbagine in protecting the DNA against CA–induced oxidative
416 damage. The results show that electrochemical impedance spectroscopy and cyclic
417 voltammetry are the best tools for quantitative comparison of different reagents for DNA
418 damage. **"Here Fig. 6"**
419 *3.7. Investigation of dopamine and resorcinol mediated DNA damage using*
420 *DNA/CA@MWCNT/PGE*
421 To investigate the influence of catechol derivatives on the DNA damage, we have
422 repeated the electrochemical procedure with other dihydroxy systems, containing
423 dopamine (DA, 3,4-dihydroxyphenethylamine) and resorcinol (RS, *m*-dihydroxybenzene).

Fig. 6B shows the impedance spectra of DNA/DA@MWCNT/PGE and DNA/RS@MWCNT/PGE before and after immersing in Tris buffer solution (pH 7.0) containing 20.0 μmol L^{-1} Cu^{2+}. As shown in this figure the amount of Rct was decreased after the incubation. This observation was not seen after incubation of DNA/DA@MWCNT/PGE and DNA/RS@MWCNT/PGE in Tris buffer solution (pH 7.0) without any Cu^{2+}. From this extended study on the dopamine and resorcinol mediated DNA damage using proposed DNA biosensor (Fig. 6B), it has been concluded that this strategy could be a suitable method for detection of catecholics (catechol derivatives) mediated DNA damage.

3.8. General comments

When catechol is oxidized enzymatically or in the presence of oxygen and heavy metals ions, one electron is transferred to the molecular oxygen, and consequently a superoxide ($O_2^{\bullet}$) is formed. In the presence of heavy metals (e.g., copper, iron), the superoxide is further reduced to hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$). CA can act as pro-oxidant, and as a consequence of its redox cycling activity may damage macromolecules such as DNA and proteins and destroy membrane function (Schweigert et al., 2001a; Schweigert et al., 2001b). DNA strand breaks are caused by the redox reaction of Cu(II) and catechol to yield Cu(I) and the semiquinone radical, and the subsequent copper catalyzed reduction of molecular oxygen to form superoxide and hydrogen peroxide. A DNA–copper–ox complex [DNA–Cu(I)–OOH] finally causes the DNA strand breaks by splitting hydroxyl radicals in the vicinity of the DNA (Schweigert et al., 2000; Sonntag, 1987).

Related to this, the OH released from a bound hydroxyl radical immediately attacks an adjacent constituent of DNA, before it can be scavenged by the OH scavengers

(Sonntag, 1987) Glutathione and plumbagin are two examples of antioxidants that scavenge OH radicals.

4. Conclusion

We have demonstrated that the preparation of catechol functionalized DNA biosensor for direct monitoring and assessment of catechol mediated DNA damage. By applying EIS as an analytical technique, assessment of DNA damage in different matrixes and comparison of different reagents on the catechol mediated DNA damage investigated. The experimental results obtained in this study suggest that DNA damage is highly affected by the concentration of the transition metals and the incubation time. Finally, the lowest concentrations of several metals ions for CA induced DNA damage were also compared. This work proves that the influence of different reagents on the reaction rate of DNA damage could be studied by electrochemical methods. While the concept has been illustrated for the CA mediated DNA damage, it could be readily extended to monitor the CA derivatives induced DNA damage with encapsulating of them on the PGE. The new direct monitoring capability of DNA damage thus offers considerable promise for characterizing the potential of DNA damage of new synthetic catecholics.

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Legends for the figures:

Fig. 1. (A) Consecutive cyclic voltammograms of 1.0 mmol L^{-1} CA obtained in 0.1 mol L^{-1} PBS (pH 7.0) at MWCNT/PGE. (B) Cyclic voltammogram of CA@MWCNT/PGE obtained in 0.1 mol L^{-1} PBS (pH 7.0). Scan rate, 50 mV s^{-1}.

Fig. 2. (A) Impedance spectra of (a) bare PGE; (b) MWCNT/PGE; (c) CA@MWCNT/PGE; and (d) DNA/CA@MWCNT/PGE in 0.1 mol L^{-1} PBS (pH 4.0) and 5.0 mmol L^{-1} $Fe(CN)_6^{3-/4-}$ containing 0.1 mol L^{-1} KCl. TEM images of (B) MWCNTs and (C) CA@MWCNTs.

Fig. 3. (A) Impedance spectra of DNA/MWCNT/PGE in 5.0 mmol L^{-1} $Fe(CN)_6^{3-/4-}$ containing 0.1 mol L^{-1} KCl in PBS (pH 4.0) as a): DNA/MWCNT/PGE was incubated in Tris buffer solution (pH 7.0) (free of CA and metal ions) with stirring time of 2 h; b): DNA/MWCNT/PGE was incubated in a CA solution (100.0 μmol L^{-1}) in the absence of the metal ions; c): DNA/MWCNT/PGE was incubated in a CA solution (100.0 μmol L^{-1}) in the presence of 20.0 μmol L^{-1} Cu(II), (d): 20.0 μmol L^{-1} Fe(III), and (e): 20.0 μmol L^{-1} Cr(VI) in Tris buffer solution (pH 7.0) with stirring time of 2 h. (B) Formation of 8-oxodG from salmon sperm DNA induced by catechol. 0.5 mg dsDNA was incubated with CA (100.0 μmol L^{-1}) in the presence of (a) Fe(III), (b) Cu(II), (c) Cr(VI), (d) Mn(II), (e) Co(II), (f) Ni(II), (g) Zn(II), and (h) Cd(II) with concentration of 20.0 μmol L^{-1} in Tris buffer solution (pH 7.0). (C) The dependence of the Rct of DNA/MWCNT/PGE on the CA concentration in 5.0 mmol L^{-1} $Fe(CN)_6^{3-/4-}$, 0.1 mol L^{-1} PBS (pH 4.0) and 0.1 mol L^{-1} KCl. DNA/MWCNT/PGE was incubated in the CA solution with different concentrations in the presence of different transition metals (Cu(II), first bars; Fe(III), 2nd bars and Cr(VI) 3rd bars) with stirring time of 2 h.

559 **Fig. 4.** (A) Impedance spectra of DNA/CA@MWCNT/PGE in 5.0 mmol L^{-1} $Fe(CN)_6^{3-/4-}$
560 containing 0.1 mol L^{-1} KCl in PBS (pH 4.0). DNA/CA@MWCNT/PGE was incubated in
561 Tris buffer solution (pH 7.0) in the (a): absence and presence of 20.0 µmol L^{-1} (b) Cu(II),
562 (c): 20.0 µmol L^{-1} Fe(III), and (d): 20.0 µmol L^{-1} Cr(VI) with stirring time of 2 h. (B) The
563 dependence of the Rct of DNA/CA@MWCNT/PGE on the transition metal concentration
564 in 5.0 mmol L^{-1} $Fe(CN)_6^{3-/4-}$, 0.1 mol L^{-1} PBS (pH 4.0) and 0.1 mol L^{-1} KCl.
565 DNA/CA@MWCNT/PGE was incubated in Tris buffer solution (pH 7.0) in the presence
566 of different transition metals (Cu(II), first bars; Fe(III), 2nd bars and Cr(VI) 3rd bars) with
567 different concentrations and with stirring time of 2 h. (C) The dependence of the Rct of
568 DNA/CA@MWCNT/PGE on the incubation time of DNA/CA@MWCNT/PGE in
569 transition metals in 5.0 mmol L^{-1} $Fe(CN)_6^{3-/4-}$, 0.1 mol L^{-1} PBS (pH 4.0) and 0.1 mol L^{-1}
570 KCl. DNA/CA@MWCNT/PGE was incubated in Tris buffer solution (pH 7.0) in the
571 presence of (a) Cu(II), (b) Fe(III) and (c) Cr(VI)) with different stirring time.

572 **Fig. 5.** Effect of different water matrices on the charge transfer resistance of
573 DNA/CA@MWCNT/PGE after 2 h incubation in the samples. pH of samples were
574 reached to 7.0.

575 **Fig. 6.** (A) The effect of glutathione and plumbagin on the Rct of
576 DNA/CA@MWCNT/PGE in 5.0 mmol L^{-1} $Fe(CN)_6^{3-/4-}$, 0.1 mol L^{-1} PBS (pH 4.0) and
577 0.1 mol L^{-1} KCl. DNA/CA@MWCNT/PGE was incubated in Tris buffer solution (pH
578 7.0) in (a): absence and presence of (b) 20.0 µmol L^{-1} Cu(II) and (c) 20.0 µmol L^{-1} Cu(II)
579 and 20.0 µmol L^{-1} glutathione and (d) 20.0 µmol L^{-1} Cu(II) and 20.0 µmol L^{-1} plumbagin
580 with stirring time of 2 h. (B) Impedance spectra of (a and c) DNA/DA@MWCNT/PGE
581 and (b and d) DNA/RS@MWCNT/PGE in 5.0 mmol L^{-1} $Fe(CN)_6^{3-/4-}$ containing 0.1 mol
582 L^{-1} KCl in PBS (pH 4.0). DNA/DA@MWCNT/PGE and DNA/RS@MWCNT/PGE were

583 incubated in Tris buffer solution (pH 7.0) in the absence (a and b) and presence of 20.0
584 μmol L^{-1} Cu(II) (c and d) with stirring time of 2 h.

585 ◆ Catechol encapsulated on MWCNTs on a pencil graphite electrode was prepared.

586 ◆ DNA damage induced by radicals generated from catechol was characterized.

587 ◆ Electrochemical method was used to direct monitor of genotoxicity of catechol.

588 ◆ The presence of Cu(II), Cr(VI) and Fe(III) on the radicals generated from catechol was
589 studied.

590 ◆ Inhibitory effect of glutathione and plumbagin on the catechol mediated DNA damage was
591 investigated.

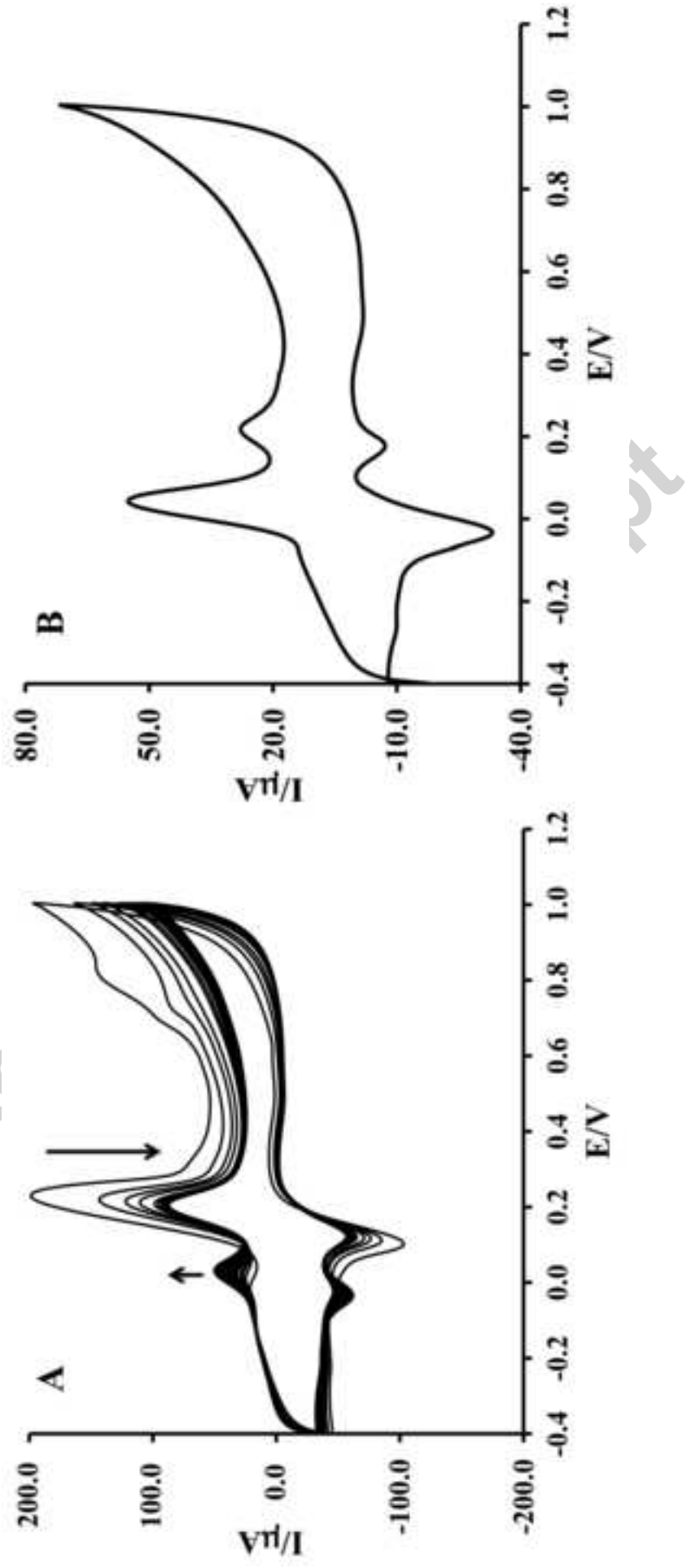
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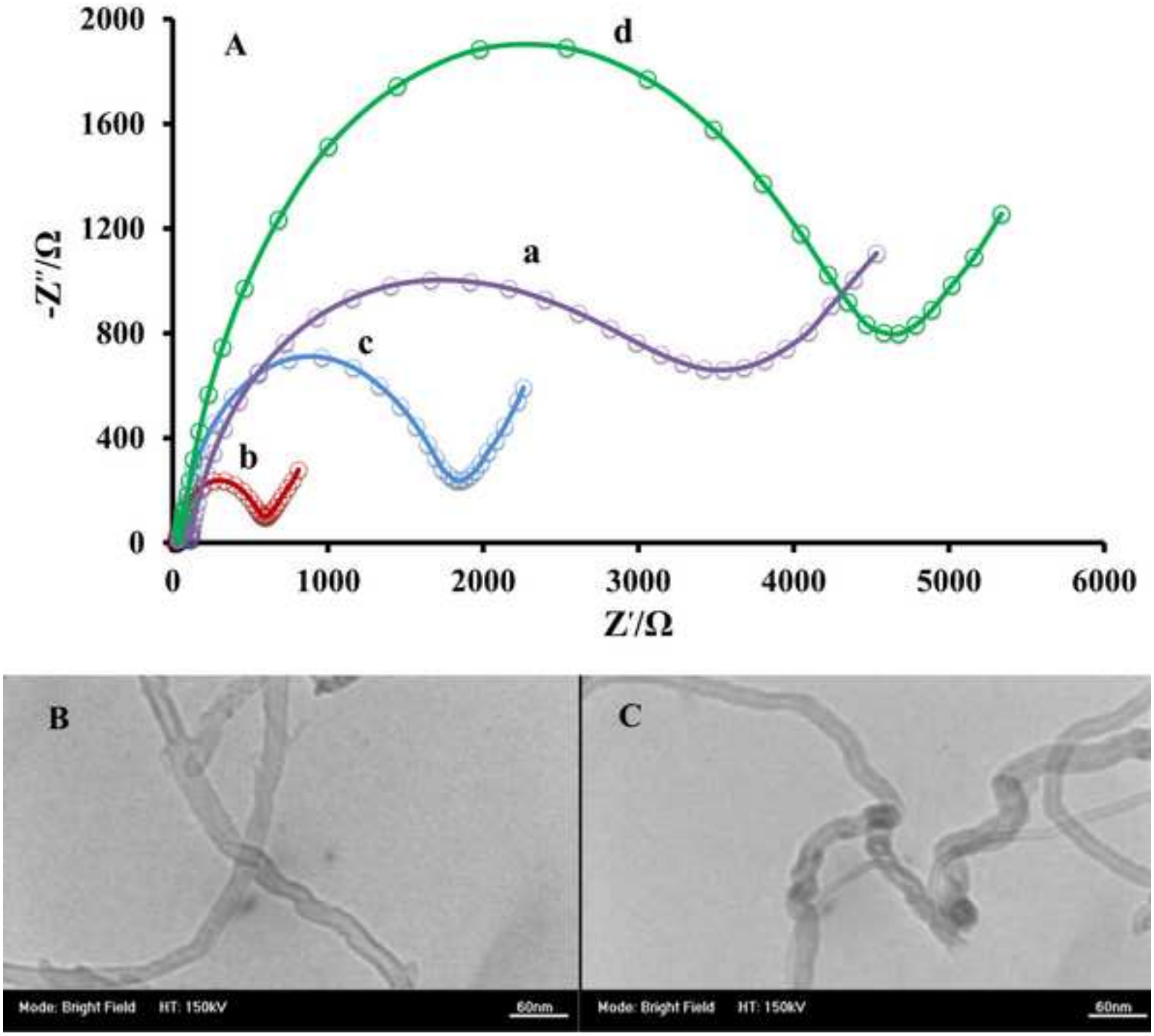
Figure 1

Figure 2



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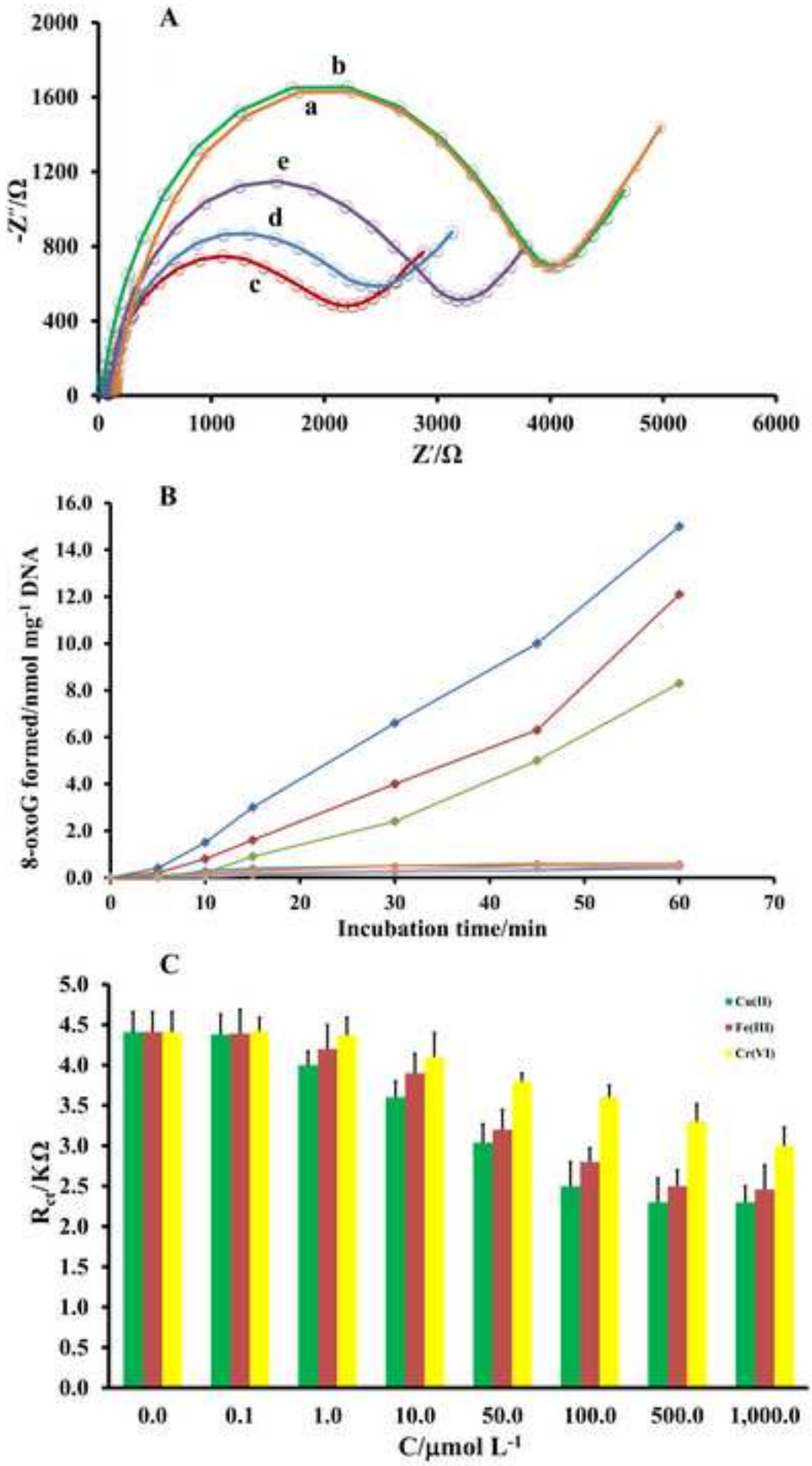
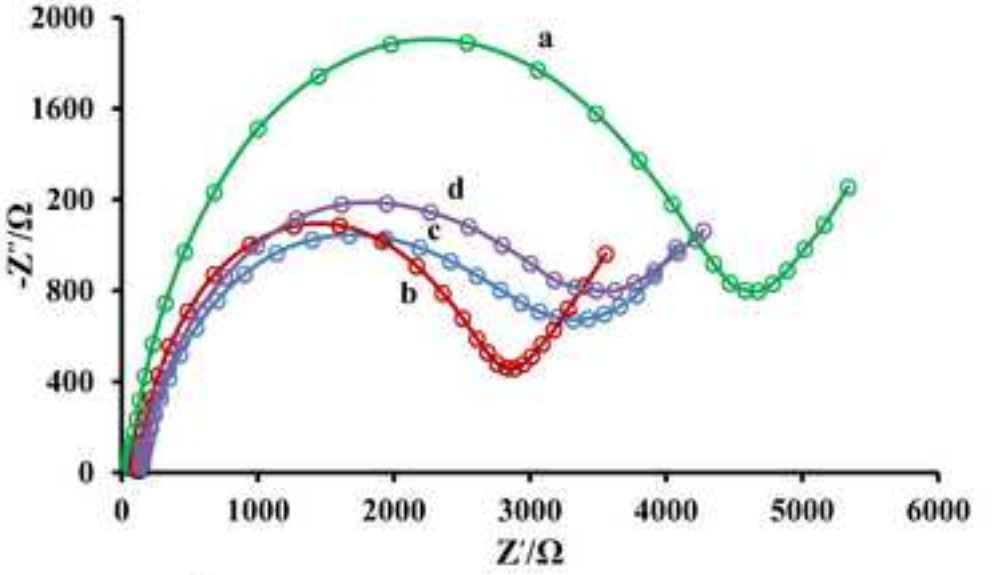
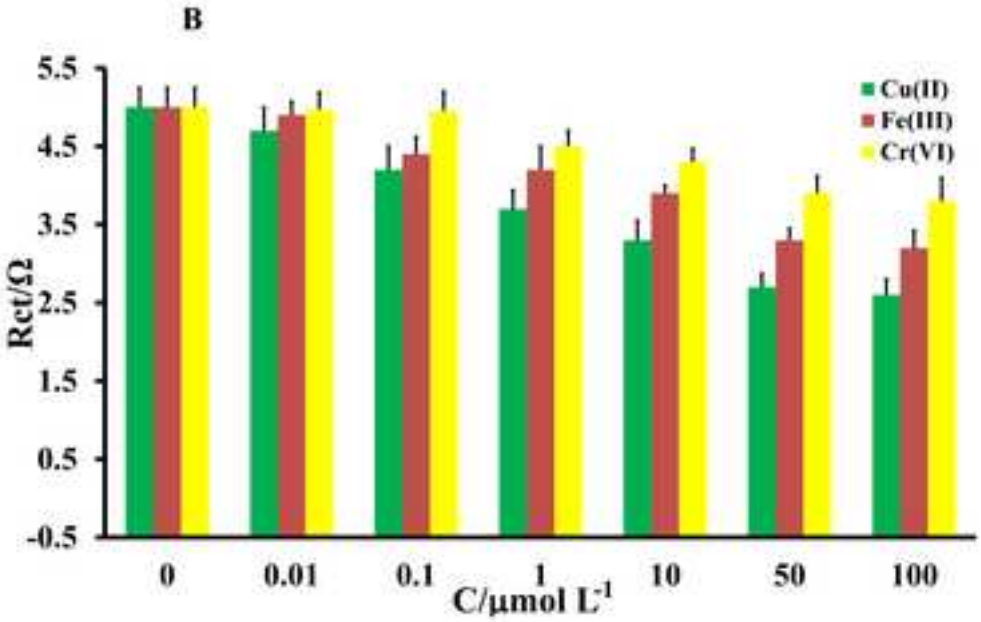
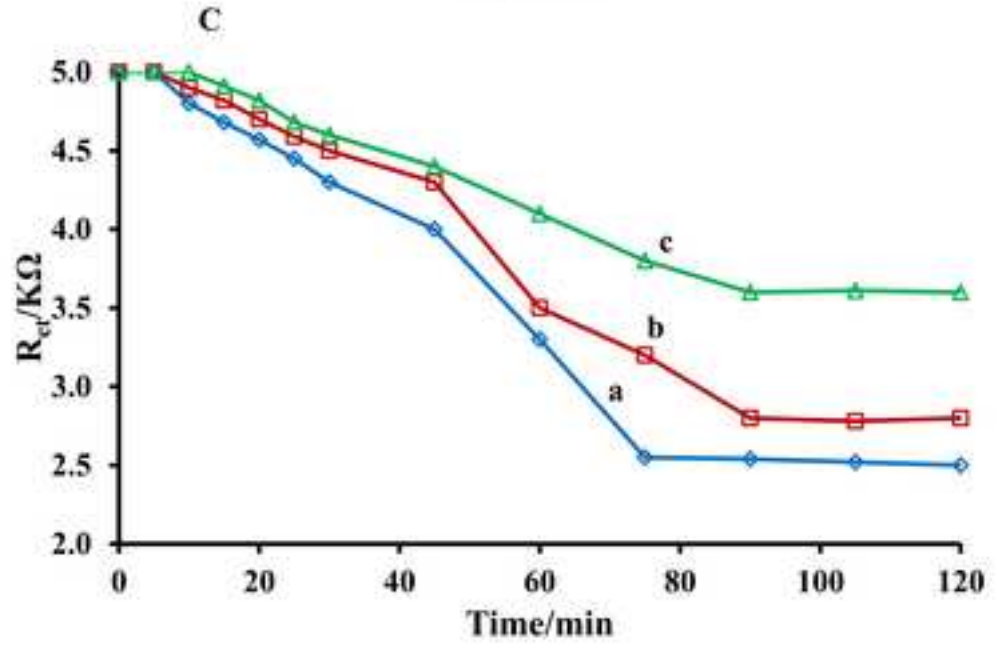


Figure 4







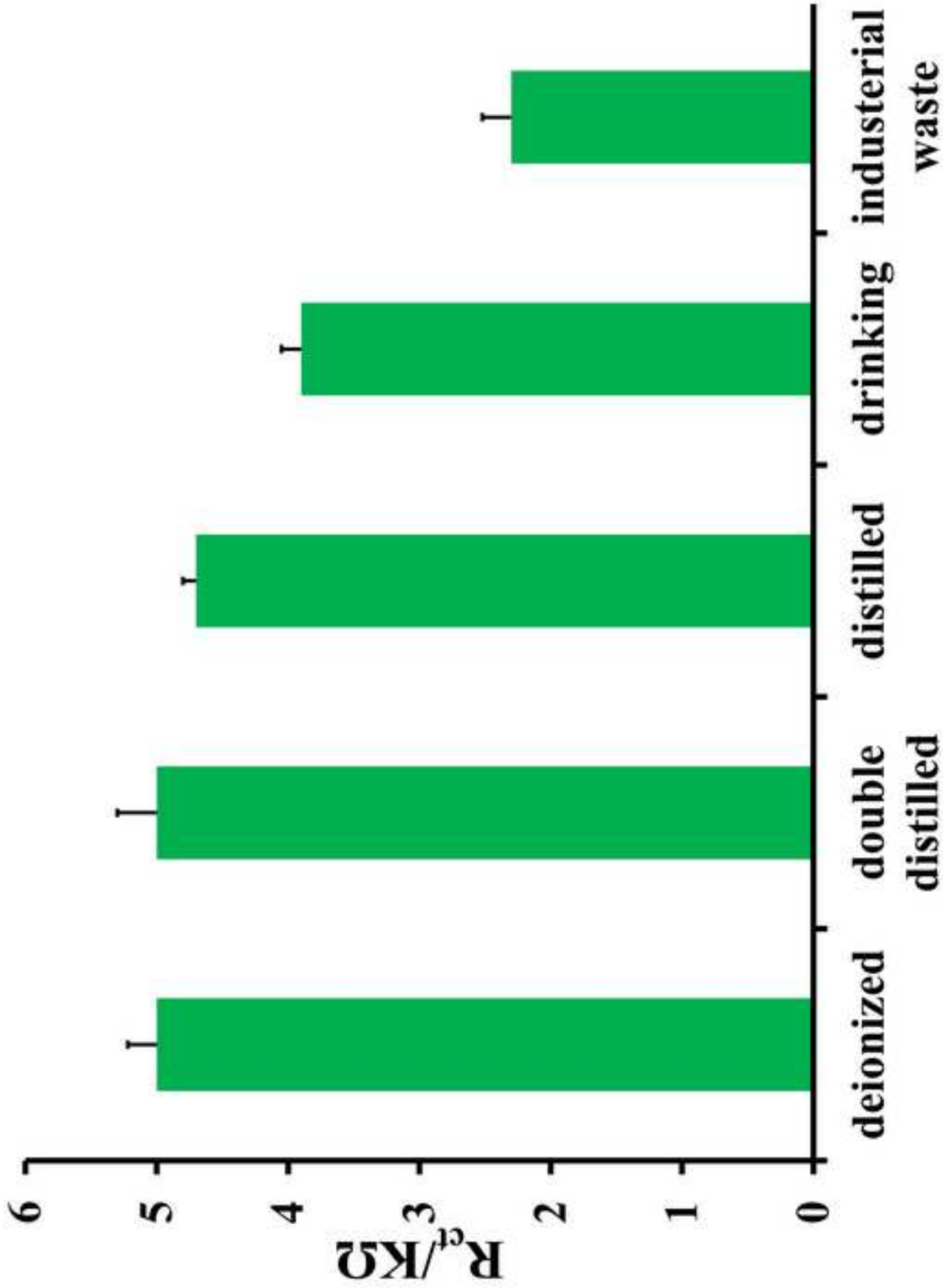


Figure 5

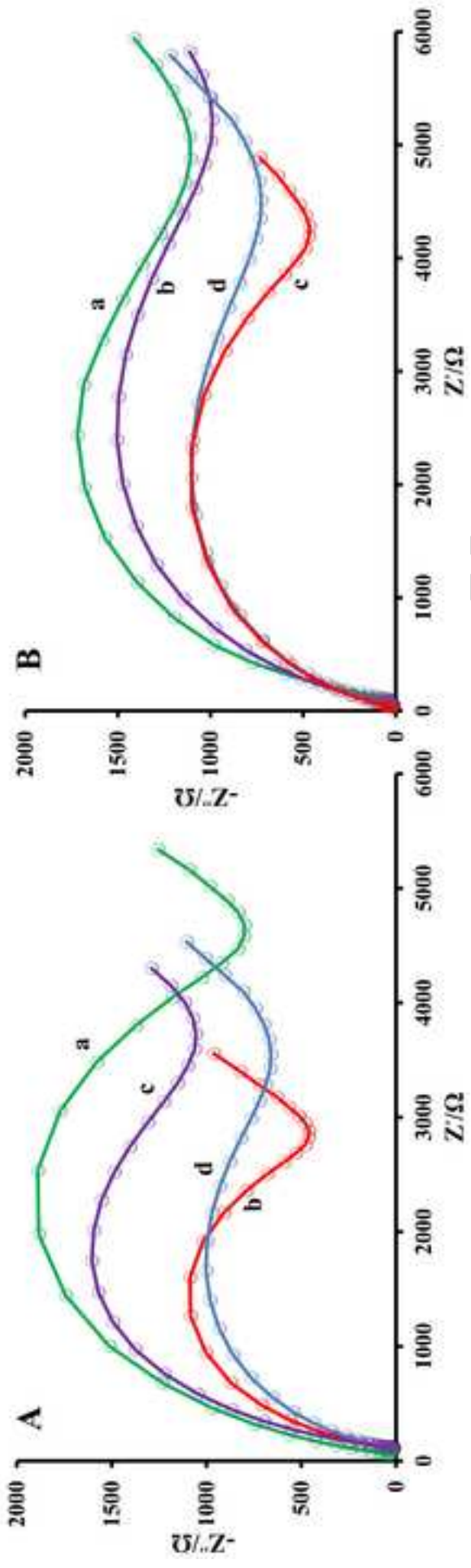


Figure 6