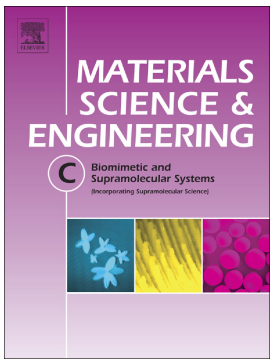

A novel simple biosensor containing silver nanoparticles/propolis (bee glue) for microRNA let-7a determination

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A novel simple biosensor containing silver nanoparticles/propolis (bee glue) for microRNA let-7a determination

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Abstract

A novel sensitive electrochemical sensor for microRNAlet-7a detection in normal serum samples, hepatocellular carcinoma patients and human liver cancer cells, has been excellently synthesized. The sensor constructed of carbon paste (CP) amended with silver nanoparticles (AgNPs) and extracted propolis (bee glue). The AgNPs/P modified carbon paste electrode (APCPE) displayed a high electrocatalytic activity in a Britton Robinson (BR) buffer (pH = 7.4). The techniques utilized to prepare this work are square wave voltammetry (SWV) and electrochemical impedance spectroscopy (EIS). Surface characteristics were achieved using scanning (SEM), Fourier-transform infrared spectroscopy (FTIR), Spectrophotometer, transmission (TEM) electron microscope, energy dispersive X-ray analysis (EDX) and elemental mapping (EM) techniques. Under optimal conditions, the suggested sensor exhibits good rapid and sensible response reaching a very low detection limit of 10^{-3} femtomolar.

Keywords: MicroRNAs; Ag Nanoparticles; Propolis; TEM; EIS.

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Introduction

The progress of electrochemical nano-modified sensors has become essential [1] owing to their high surface area. Silver nanoparticles (AgNPs) have low cost, non-toxic, biocompatibility, stable and high catalytic activity aiding us to use it for biological sensing [2-4]. Propolis is a simple viscous, natural sensitive material, consisting of ~ 45% resin, 35% wax and 20% inert material [5]. It is non-toxic, biocompatible material with good adhesion [6].

MicroRNAs (miRNAs) are a family of non-protein-coding, most of their genes is found in intragenic regions or in the anti-sense orientation of genes containing their own miRNA gene sponsor and adjusting units. The expression of miRNA is generally down regulated in tumor tissues [6-8]. Down regulation of the miRNA let-7a has been concerned in cell proliferation and poor prognosis. The let 7 family is one of the best characterized miRNA families containing 13 member located at 9 different chromosomes and several body cancers contains deregulated let 7 expression. MiRNA profiling classified hepatocellular carcinoma (HCC), which is the most common type of liver cancer, into three main clusters. These results designate the significance of miRNA detection for the HCC.

The tools which are available for the detection of microRNAlet-7a were using reverse transcriptase polymerase chain reaction (RT-PCR) [9], Northern blot [10], and microarray [11], which based on traditional non-sensor amplification based molecular techniques (MTs) that employ a selective molecular probe, but not definition of integrated transducer element. Other difficulties of these tools are usually, less sensitivity, time-consuming and/or require radio-or fluorescent-Labeling and complexed equipment. Essentially, miRNA MTs require extensive sample

preparation, including amplification steps in spite, recently there was an availability to complete this steps by using the microchip gel electrophoresis using a programmed field strength gradients (MGE-PFSG system) that can separate the DNA/RNA amplification products in an automated fashion with an adequate resolution in molecular biology, particularly to the analysis of amplified-PCR products and the capability of MGE-PFSG for the ultra-fast detection of separated gene [12-14] which can decrease time-to results and introduce measurement bias.

Over the past decade, there was a need for emerging amplification for free biosensor-based techniques for miRNA assay (BTs) which provide useful characteristics for application in both fundamental research and clinical settings. In addition to develop forceful detection methods for miRNAs with elevated selectivity, sensitivity and simplicity [15], BTs have arisen as a compliment to traditional MTs. BTs combine the use of a selective molecular probe used in traditional MTs with a highly sensitive transducer design in sensors. This gives BTs the ability to achieve rapid and highly sensitive results in complex matrices, such as blood and urine, without the use of polymerase-based amplification steps. These attractive features have led to the recent development of BTs for miRNA assay.

Accordingly, electrochemical genosensors have held excessive challenge as devices appropriate for point-of-care diagnostics and multiplexed platforms for fast, easy and low-cost nucleic acid analysis [16].

The electrochemical detection of miRNAs is based on the guanine oxidation consequent to the hybridization between the target miRNA and its complementary (DNA capture probe) [17]. Propolis has excellent electrochemical property for guanine oxidation enhancing the sensitivity for its determination due to it increases biosensor surface area, resulting in improved miRNA biosensor performance.

All of the electrochemical miRNA detection ways have been accessible in the literature relying on hybridization, when it happens a signal appears. One of the limiting factors for its creation is the sensitivity [18, 19]. Based on the hybridized miRNA-templated position of an insulating polymer film, Gao et al. [20] make a simple and ultrasensitive impedimetric miRNA biosensor. The accumulative nature of the deposition process enhances well the biosensor sensitivity. Ren et al.[21] design a highly sensitive and selective miRNA biosensor by using a duplex-specific nuclease, which initiates cleavage of the hybridized target miRNA to amplify the electrochemical signal. Nanomaterial's used involve silver nanoparticles or nanoclusters [22] significantly improve the detectability of miRNAs [23]. Fan et al.[24] prepare a sensitive electric biosensor array based on the hybridized miRNA-guided insitu formation of polyaniline nanowires, leading to significant enhancement of the signal when the target miRNA is present in the sample solution.

In this paper, we proposed a simple constructive and inexpensive sensor. The propolis and AgNPs-based biosensor exhibits good analytical properties, high sensitivity, low detection limit, and good selectivity and reproducibility for miRNA-let7a detection.

2. Experimental

2.1. Materials and reagents

Propolis material is extracted from settled hives in the El aliubiya region/Egypt then dried and grinded into fine black powder. Graphite and silver nanoparticles powder, ''dispersion nanoparticles, <100 nm'' were purchased from Sigma Aldrich. Britton Robinson (BR) (CH_3COOH + H_3BO_3 + H_3PO_4) (4.0×10^{-2} M) buffer solution, the pH value was adjusted to 7.4 by 0.2 M NaOH. Ultrapure^{TM} diethyl

pyrocarbonate (DEPC) - treated water are purchased from Invitrogen Corporation (Life Technologies, Germany). All solutions are prepared using DEPC water to prevent breakdown of miRNA. All measurements are investigated at room temperature.

A synthetic desalted purified miRNA let7a, ssDNA capture probe, single base-mismatched miRNA let7a and completely mismatched sequence miRNA let7a are purchased from Eurofins, Genomic technology and service (Germany) AP Biotech.ltd.co. The base sequences of the ssDNA capture probe and the miRNAs are as follows:

miRNA probe (ssDNA probe) 5'-AAC TAT ACA ACC TAC TAC CTCA-3'

miRNA let7a (Target) 5'-UGA GGU AGU AGG UUG UAU AGUU-3'

miRNA let7a(single base mismatched) 5'-UAG GGU AGU ACG UUG UAU AGUU-3'

miRNA let7a (completely mismatched) 5'-UCC GUA GAA UUU AAG AGU UUAA-3'

The total miRNA was extracted from serum aliquots of normal persons with concentration (34.84 µg/ml and 33.33 µg/ml) respectively. Four serum aliquots for patients sorrow from hepatocellular carcinoma are obtained from (National Hepatology & Tropical Medicine Research institute in Cairo) with concentrations (27.11 µg/ml, 26.36 µg/ml, 39.49 µg/ml and 17.14 µg/ml) and from two cultivated cancerous cell lines Huh 7.5 cells and HEPG 2 cells preserved in DMEM medium (Lonza Bioproducts, Belgium) with concentration (34.15 µg/ml and 37.51 µg/ml), respectively using (QIAGEN sample and assay technologies) according to constructor's recommended protocol.

2.2. Sensor creation

The sensor was made-up [25] by adding 0.5 g graphite powder to paraffin oil drops using a morter then 10 mg of AgNPs and propolis (PP) are mixed in the paste under

strong mixing using a morter to build on the sensor. After that the teflon tube is filled by the obtained paste and pressed well then the modified electrode (CPE/AgNPs/PP) was washed with DEPC water and then dried. At this step, the electrode is ready for use.

2.3. Cell and Apparatus

The electrochemical measurements were performed by **SP-150** potentiostat supplied with **EC-Lab® software package**. A typical three-electrode cell of 25 mL volume was used with a platinum rod as a counter electrode (CE), saturated calomel electrode (SCE) as a reference electrode (RE) and (APCPE) as the working electrode (WE). A microprocessor digital pH-meter (Hanna instruments, Italy) is used for pH measurements. Scanning electron microscopic (SEM) measurements are done by SEM Model Quanta 250 FEG (Field Emission Gun) connected to EDX Unit (Energy Dispersive X-ray Analyses) (FEI company, Netherlands). TEM images were performed by JEM-2100 (high-resolution transmission electron microscope) (HRTEM). FT-IR spectrum were performed by Agilent technologies Cary 630, while UV measurements were performed using a Shimadzu 1601 spectrophotometer (Kyoto, Japan).

3. Results and discussion

3.1. Surface characteristics of APCPE biosensor

The sensor morphology is observed as shown in Fig. 1A as SEM which shows equal distribution of the Ag nanoparticles together with propolis on the electrode surface with average size of ~ 35 for AgNPs as gotten from TEM micrographs (Fig 1B). EDX (Fig. 1C) ensures the presence of both silver nanoparticles and propolis with uniform distribution as confirmed by elemental mapping (Fig. 1D).

Insert Figure 1

The scheme for the preparation of the modified sensor containing AgNPs/propolis and capture probe immobilization is shown on Fig 1E. It shows the way where propolis containing silver nanoparticles adsorbed on the electrode surface with its sticky properties[5]. Also, it has porous structure that can increase the surface area of the sensor and highly conductive facilitating the charge transfer process.

After the addition of AgNPs/P on the electrode surface with large surface area, the sensor was used to detect microRNA let-7a with high sensitivity, where microRNA probe is immobilized on the biosensor surface through propolis. This makes microRNA stable on the sensor surface. So, the sensor works well giving high sensitivity.

The spectrophotometric spectrum of silver nanoparticles gives a characteristic absorption peak at 410 nm which indicates the presence of Ag nanoparticles as given in Figure 2. Also, FTIR spectrum of APCPE was performed (Figure 3) where silver nanoparticles display an absorption band at 1640 cm^{-1} due to the stretching of C=O bonds while propolis displays absorption band at 1180 cm^{-1} due to the stretching between the aromatic ring and C-H of propolis, and other two absorption bands at 2150 and 3400 cm^{-1} due to the stretching of C≡C and O-H bonds respectively. This insures the reaction between propolis and silver nanoparticles.

Insert Figure 2,3

3.2.*Optimization of Biosensor settings*

Optimization of the surface design for the sensor has been done to create a highly stable and reproducible genosensor. A room temperature of 25 ^{o}C is selected as the operative temperature for the utilized sensor, where the increase in the current response is attained at this temperature (Fig 4A) owing to denaturation of proteins with rising temperature [26]. Also, the effect of incubation time has been performed

where a constant value has been attained at the time of 30 minutes (Fig. 4B). So, 30 minutes incubation time was selected, to allow hybridization to proceed in all experiments.

3.3. *Electrochemical detection*

Insert Figure 5

The electrochemical oxidation of miRNA-let 7a on the created APCPE biosensor surface was evaluated by electrochemical impedance spectra and square cyclic voltammetry within the potential range of 650 mV to 950 mV, in BR buffer of pH 7.4 (Fig 5A,B), respectively. The detection was depending on the charge transfer resistance and/or the current response change before (Target) and after hybridization (Target + Probe). The peak current at 796 mV arises from the charge transfer between silver nanoparticles and PP with the hydroxyl group at the 2' position of the ribose sugar and/or with Guanine lone pair of electrons on nitrogen, which facilitated the charge transfer process. The impedance value (Fig.5A) increases for miRNA-let 7a on immobilizing the probe onto the biosensor surface with a noticeable decrease in the peak current (Fig. 5B) owing to a formation of less conducting film that hinders the charge transfer process [25, 27]. Finally, the peak current vanishes after the addition of the target miRNA-let7a (single base mismatched) and the (complete mismatched), and the impedance value increases, respectively, on introducing a hybridization disorder between the probe and the target, leading to a weak interactions that hinder the electrons transmission towards the surface. The Nyquist diagrams were fitted using a two parallel constant model presented as inset in Fig.6. It consists of R_s as the solution resistance, R_1, R_2 as both external and internal charge transfer resistance. CPE_1 and CPE_2 are the corresponding constant phase elements (CPE) for both

external and internal layers instead of ideal capacitances owing to non-homogenious biosensor surface [28, 29].

Insert figure 6

The APCPE sensor was immersed for 30 minutes before each measurement, in the BR buffer type containing synthetic miRNA let7a (target+Probe) solutions with different concentrations for the target in the range of 10^{-3} femtomolar to 1.0 micromolar. This was done to allow hybridization to proceeds for 30 minutes after successive washing with DEPC water in turn for 5 minutes. The electrochemical oxidation at the surface of modified sensor containing AgNPs/Propolis was investigated using electrochemical impedance spectra in BR buffer at the peak potential at 796 mV as shown in Figure 5. It was found that the semicircle diameter increases with increasing the concentration indicating an increase in impedance value [30]. A fitting was done for the Nyquist curves with the model shown as inset in Fig. 6.

To examine the viability and the sensitivity of the created biosensor, the relationship between the ($Z-Z^o$) and the logarithm of miRNA-let 7a concentration was drawn (Fig 6). The guanine oxidation signal of the probe/modified biosensor was measured as a background and Z^o is its value and subtracted from all the measured data (Z). It was found that ($Z-Z^o$) increases with miRNA-let 7a concentration (from 10^{-3} femtomolar to 1.0 micromolar) owing to the interaction between the DNA probe and the miRNA-let 7a that produces an alteration in the probe orientation and afford a better approachability of the probe and therefore improve the hybridization efficiency at the sensor's surface, and accordingly the ($Z-Z^o$) increases.

The regression equation is: $(Z-Z^o) = 5.214 + 0.2996 \log C_{miRNA\text{-}let7a}$ with a correlation coefficients of 0.993, and the limit of detection is 10^{-3} femtomolar.

3.4. Real samples applications

The analytical applicability of prepared probe/CP/AgNPs/propolis electrode for detection of miRNAlet7a was performed in extracted miRNA samples from different serum samples collected from normal persons and HCC patients, also from hepatic cancerous cultured cell lines.

The most common primary liver cancer is hepatocellular carcinoma HCC, so, it is important to be studied. The application of the created probe/CPE/AgNPs/PP biosensor for the discovery of miRNAlet7a was achieved on extracted miRNA trials from different serum aliquots collected from two normal persons (Fig 7A), four HCC patients (Fig 7B) and two hepatic cancerous cultured cell lines Huh7 and HepG2 gotten from human liver tumor (Fig 7C and D respectively) before and after hybridization. All tests are done in compliance with the relevant laws and institutional guidelines, and have been approved by the institutional committee. The obtained data informs that miRNA-let 7a current response of normal persons (control) is lower than that of the four HCC patients, owing to miRNA expression is generally down regulated in tumor tissues compared to the normal tissues. This shows that the presence of a subset of miRNA can act as tumor suppressors like miRNA-let 7a [31], where the impedance responses of patient 2 and 3 are lower than patients 1 and 4 as they both suffer also from virus C (Fig 7B) owing to HCV specific miRNAs that potentially regulate viral replication and host gene signaling pathway on the other hand presence of miRNAs like miRNA let 7a which is one of the miRNA make down regulation for HCV patients as it has been reported from the human endogenous miRNAs involved in defense mechanism mainly against RNA viruses. However, (Fig 7 C and D) shows the lowest impedance miRNA-let 7a response due to the presence of the liver tumor that increases the replication disorder in the miRNA and thus

decreases the impedance value. These results indicated that the prepared miRNA biosensor can perform an auspicious analytical investigation in real biological samples.

Insert figure 7

3.5. Selectivity of APCPE biosensor

The selectivity of APCPE biosensor was evaluated by the electrochemical impedance detection of miRNA let 7a in the presence of single base mismatched and completely mismatched miRNA let 7a in BR buffer of pH 7.4. The impedance value for miRNA-let 7a didn't change in presence and absence of the interferences indicating the selectivity of the method.

3.6. Comparison of the efficiency of the method with the reported methods

Comparisons of the data gotten for microRNA let-7a determination by numerous methods are publicized in Table 1 [32-35], respectively. The utilized sensor in this work is of higher selectivity and sensitivity relative to others with simple way and instruments.

Table 1
Comparison of the projected APCPE sensor with others for electrochemical determination of microRNA platform.

Method	Detection limit	Ref.
Amperometry using glucose oxidase detection probe	10 FM	[32]
CVand amperometry based on enzyme amplification	3 FM	[33]
Carbon nanotube-based label-free electrochemical biosensor	1 pM	[34]
Chronoamperometry using capture LNA molecular beacons	0.06 PM	[35]
APCPE sensor	10^{-3} FM	This work

4. Conclusions

APCPE as an electrochemical biosensor for microRNAs detection in hepatocellular carcinoma has shown a very low detection limit reaching to 10^{-18} and a high sensitivity. Good acceptable results have been obtained for miRNA-let 7a determination in hepatocellular carcinoma patients and/or in hepatic cancerous cultured cell lines Huh7 and HepG2. This ensures that this created sensor can be applied widely in several subjects.

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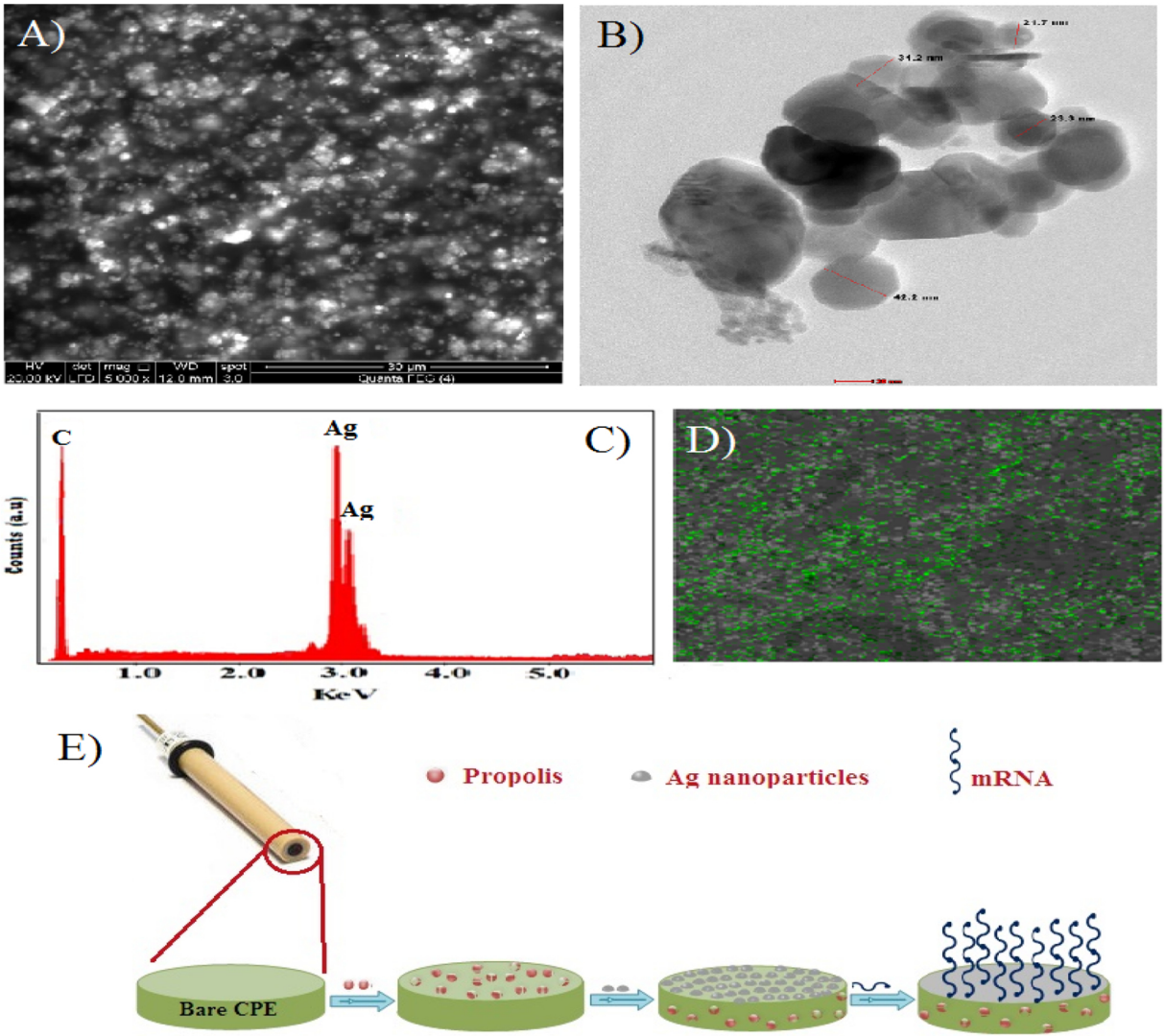
Highlights

- A novel sensitive electrochemical sensor for microRNAlet-7a detection.
- The sensor constructed of carbon paste (CP) amended with (AgNPs) and extracted propolis.
- The sensor exhibits very low detection limit of 10^{-3} femtomolar.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D)
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Propolis
Ag nanoparticles
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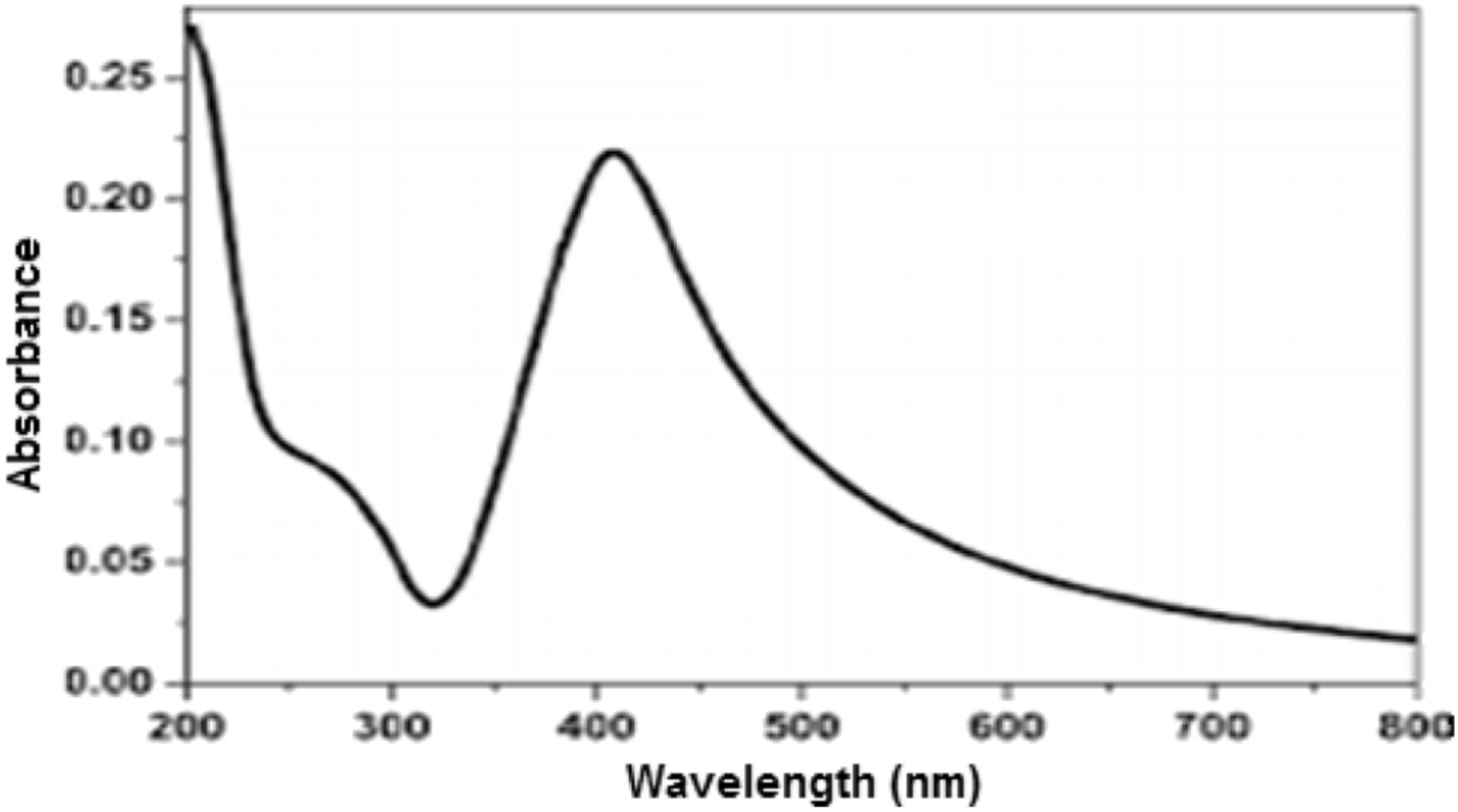
Bare CPE

Figure 1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Wavelength (nm)

Figure 2

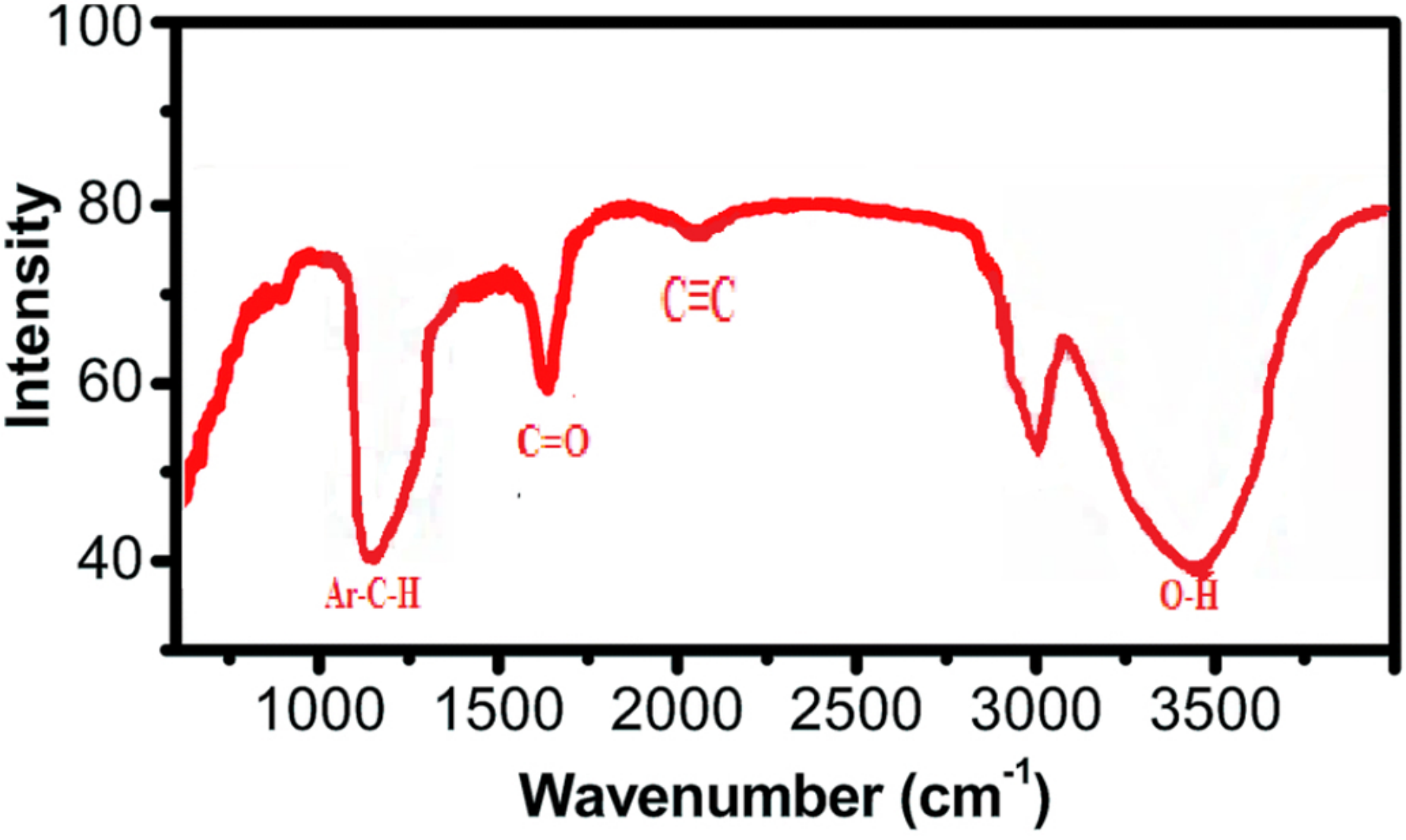
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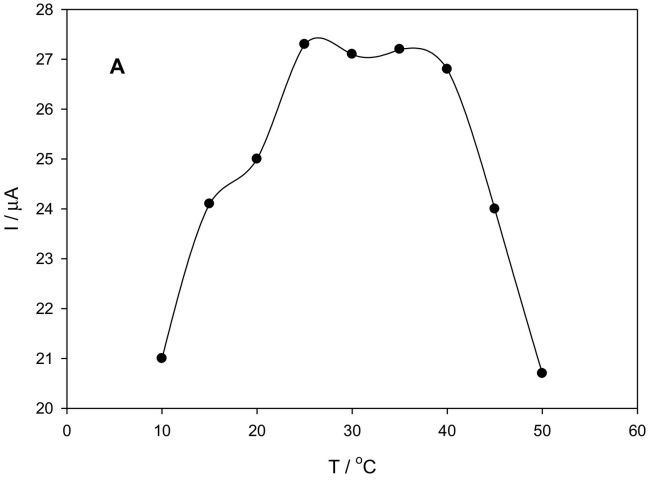
Figure 3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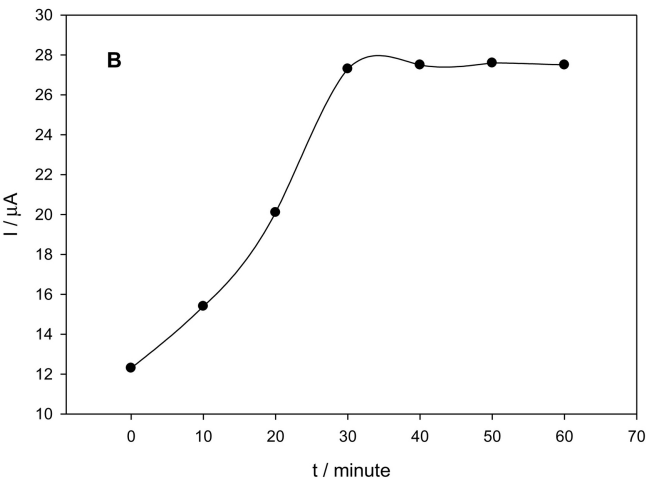
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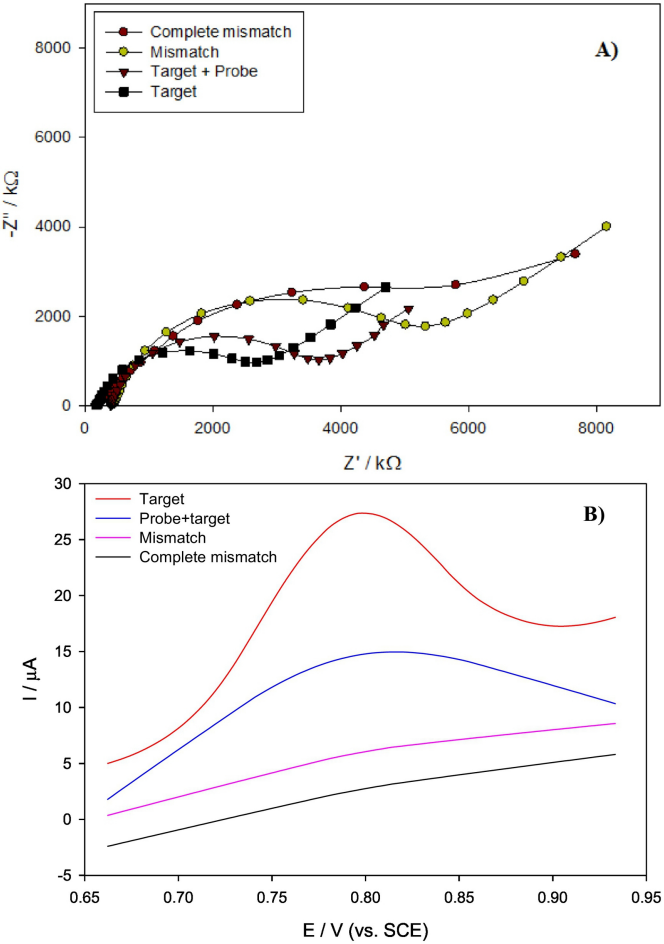
Figure 4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Complete mismatch
Mismatch
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Z' / kΩ
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6000
8000
B)
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Mismatch
Complete mismatch
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0.95

Figure 5

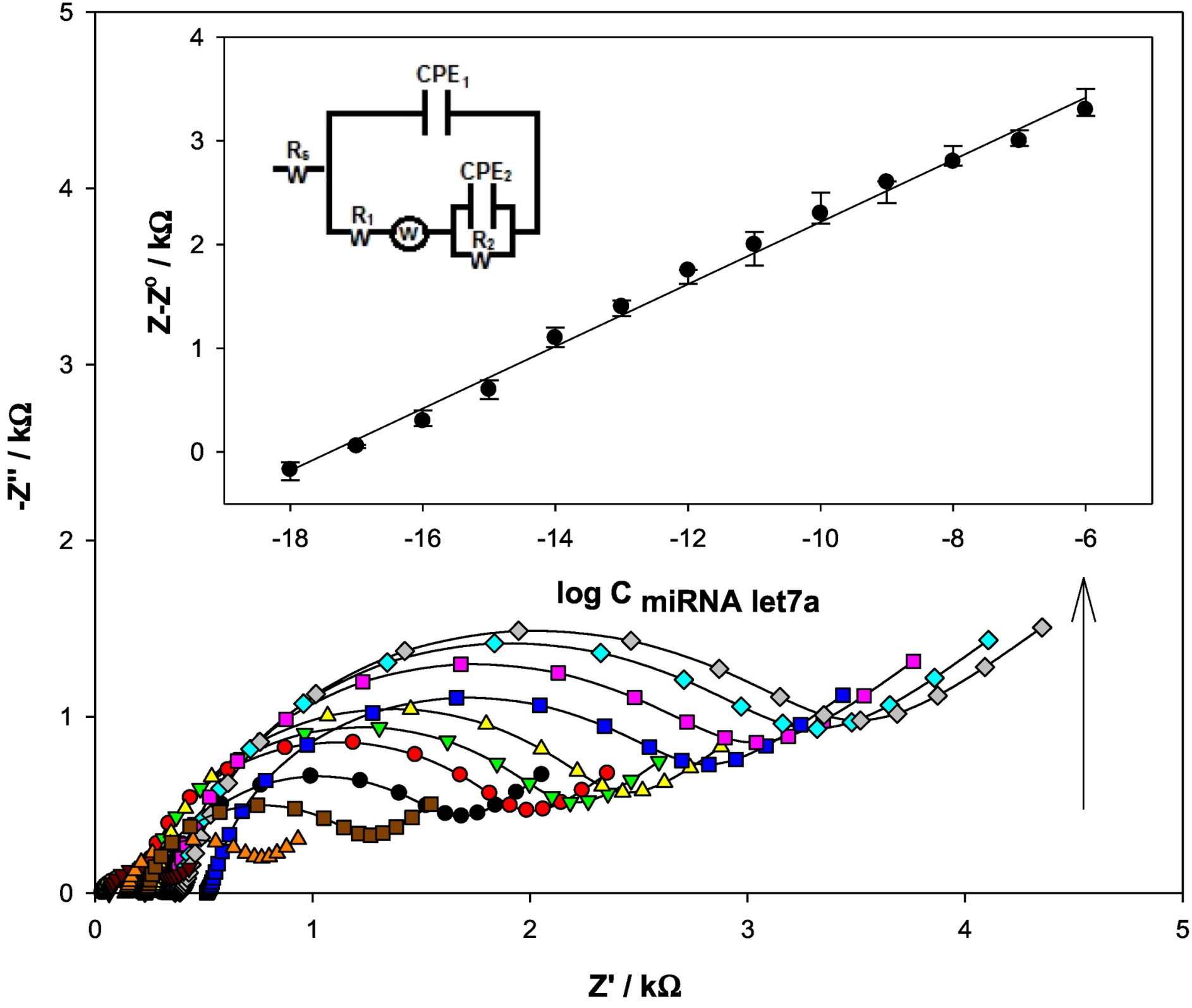

Figure 6

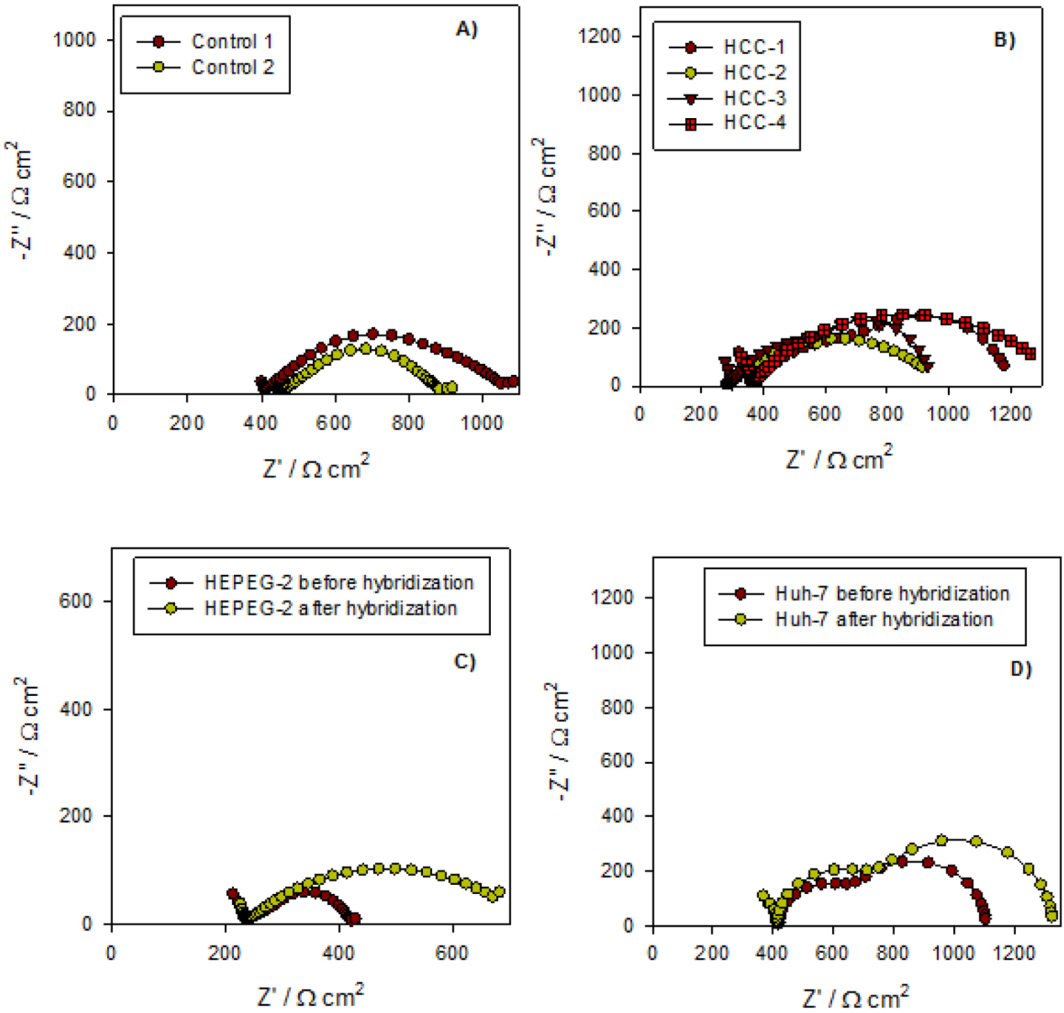
A)
Control 1
Control 2
-Z'' / Ω cm2
Z' / Ω cm2
B)
HCC-1
HCC-2
HCC-3
HCC-4
-Z'' / Ω cm2
Z' / Ω cm2
C)
HEPEG-2 before hybridization
HEPEG-2 after hybridization
-Z'' / Ω cm2
Z' / Ω cm2
D)
Huh-7 before hybridization
Huh-7 after hybridization
-Z'' / Ω cm2
Z' / Ω cm2

Figure 7