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# Paper-based electrode assemble for impedimetric detection of miRNA

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## Abstract

In the present work, a paper-based electrode assemble was developed and implemented to detect target microRNA 155 (miRNA 155) via electrochemical impedance spectroscopy (EIS) measurements. In this concept, gold nanoparticles (AuNPs) modified paper based electrode assemble system (AuNP-PE) was designed, and characterized by scanning electron microscopy (SEM), cyclic voltammetry (CV) and EIS measurements. The impedimetric detection of miRNA 155 was performed by measuring the fractional change at the charge transfer resistance ( $R_{ct}$ ). The detection limits were found as 33.8 nM in PBS and 93.4 nM in fetal bovine serum (FBS) medium, respectively. The selectivity of the proposed assay was tested against to non-complementary (NC) and mismatch (MM) miRNA sequences in the presence of mixture sample containing miRNA:NC (1:1) and miRNA:MM (1:1) in PBS (pH 7.40) or FBS. The analytical performance and the selectivity of impedimetric biosensor were also tested in FBS.

**Keywords:** paper based electrode; microRNA; gold nanoparticles; electrochemical impedance spectroscopy.

## 31 **1. Introduction**

32 microRNAs have significant roles in the regulation of differentiation, angiogenesis,  
33 proliferation, immune cell function, wound healing and apoptosis [1-4]. Especially the level of  
34 expression of Circulating miRNAs is tissue specific and have potential for use as biomarkers  
35 [2,3]. The overexpression of miRNA-155, which is known as a significant circulating miRNA,  
36 can be used as a biomarker for diagnosis and progression of cancers [5,6]. microRNAs detection  
37 in biological systems is still challenging [7-9] and upto date, several methods are reported s  
38 for miRNA detection such as RT-PCR [10-12], microarray technique [13,14], northern blotting  
39 [15,16], and in situ hybridization [17-19]. In spite of the advantages, these methods require  
40 expensive instruments and reagents, labelled miRNAs, high sample volumes and relatively pure  
41 miRNA samples [20]. Therefore, it is extremely important that miRNA detection methods  
42 should be simple, fast and label-free [21]. At this juncture, electrochemical techniques have  
43 received notable attention due to their easy controllability, good stability, reproducibility,  
44 sensitiveness and cost-effectiveness [22].

45 Currently, various nanostructure-based sensing platforms have been employed in constructing  
46 DNA sensors owing to nanomaterial's unique advantages (e.g., large surface areas, unique  
47 properties such as mechanical, electronic and catalytic) [23]. Among the nanomaterials, gold  
48 nanoparticles (AuNPs) have become interesting option for biomedical use due to their several  
49 kinds of effective properties [24,25]. In contrast to the graphene and iron oxides, the high  
50 affinity of the gold-sulfur bonding and other unique properties, such as high conductivity and  
51 large surface [26-28]. AuNPs, with the diameter of 1-100 nm, have high surface-to-volume  
52 ratio and high surface energy to provide a stable immobilization of a large amount of  
53 biomolecules retaining their bioactivity. Additionally, AuNPs are able to allow fast and direct  
54 electron transfer between electroactive species and electrode materials [29]. A label-free and  
55 simple electrochemical microRNA biosensor was developed by Tian et al. (2018). Toluidine  
56 blue (TB) and AuNPs superlattice were used as a redox indicator and support material,  
57 respectively. The polypyrrole coated AuNPs was self-assembled n order to obtain the maximum  
58 current. Under optimum conditions, the hybridization of single strand RNA probe with the  
59 miRNA target sequence were monitored using CV and differential pulse voltammetry (DPV)  
60 by measuring the peak current of TB [29]. In another work, DNA hybridization was investigated  
61 using AuNPs and DNA immobilized GCE. Thiol linked probe was immobilized onto GCE and  
62 the hybridization of probe and target DNA was examined using DPV technique by measuring

63 the Methylene blue signal [30]. A simple filtration based assay in order to develop paper based  
64 biosensor was performed by Tian et al. [31] for simultaneous detection of miRNA 144 and  
65 miRNA 21. MOF conjugated bio-probe, methylene blue (MB) and ferrocene (Fc) were used for  
66 contribution of sensor sensitivity [31].

67 Polymers and paper are commonly used flexible substrates for the development of bioanalytical  
68 devices [8,9,32-35]. Particularly, paper is flexible, widely available, cost-effective, hydrophilic,  
69 easy to demolish and biocompatible. Therefore, it is a convenient substrate for biosensors.  
70 Additionally, the adsorption of biomolecules or nanoparticles can be performed easily due to  
71 the porosity of the paper surface [32-35]. Paper based microfluidic electrochemical DNA  
72 biosensor was developed for the detection of Epidermal growth factor receptor (EGFR)  
73 mutations in saliva samples by Tian et al. (2017). ssDNA was adsorbed onto the surface of  
74 polypyrrole membran modified Au electrode, and the electrochemical signals were obtained.  
75 The horseradish peroxidase (HRP) recognized the methylene blue labeled DNA and showed  
76 unique electrocatalytic behavior to H<sub>2</sub>O<sub>2</sub> [36]. In another study, paper-based analytical devices  
77 ( $\mu$ PADs) in combination with screen printed electrodes were developed by Lu et al. (2012).  
78 Thionine (TH) bound to double strand DNA (TH/D1) and complementary ssDNA (S3)  
79 immobilized on nanoporous gold (NPG) and formed S3-TH/D1-NPG conjugates. The designed  
80  $\mu$ PADs nucleic acid sensor showed good performance in human serum assay [37].

81 To best of our knowledge, AuNPs modified paper electrode assemble system (AuNP-PE) was  
82 developed and applied for the first time herein for impedimetric detection of miRNA 155. After  
83 thiol linked DNA probe immobilization on AuNP-PE, its complementary target miRNA was  
84 recognized by capturing probe at the electrode surface during hybridization process. The  
85 selectivity of paper based biosensor specific to target miRNA (i.e, miRNA 155) was tested  
86 against to non-complementary (NC) and mismatch (MM) miRNA sequences, in the presence  
87 of mixture samples containing miRNA:NC (1:1) and miRNA:MM (1:1). In addition, the  
88 analytical performance and the selectivity of impedimetric biosensor were also tested in fetal  
89 bovine serum (FBS) medium.

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## 94 **2. Experimental**

### 95 ***2.1. Instruments***

96 Electrochemical measurements were performed with AUTOLAB-302 PGSTAT with NOVA  
97 (version 1.1.2 Eco Chemie, The Netherlands) software package (Eco Chemie, The Netherlands)  
98 in a Faraday cage.

### 99 ***2.2. Chemicals***

100 Carbon paste was purchased from Daejoo Electronic Materials (Shanghai, South Korea).  
101 Silver/Silver chloride (Ag/AgCl) paste was purchased from Gwent Group (Torfaen, UK). The  
102 miRNA-155 specific DNA probe, miRNA-155 target and the other oligonucleotides (see  
103 supporting information for more information) were purchased from TIB Molbiol (Germany).  
104 All other chemicals were purchased from Sigma and Merck.

### 105 ***2.3. Fabrication of paper based electrodes***

106 The most proper construction was provided using NC membrane as a structure. The printing of  
107 the hydrophobic barriers was occurred onto the NC membrane surface by using a wax printer  
108 (XEROX ColorQube™ 8570 (Norwalk, USA) after designing a pattern which includes a  
109 microfluidic channel and a working area. To provide capillary flow, channels were designed  
110 with a diameter of 2 mm and a length of 1.5 cm. In the present work, electrodes are placed in  
111 the working area and this area was designed as about 20 mm<sup>2</sup> with 270 angles to provide the  
112 maximum spread speed of liquid. The fabrication process of a paper electrode is shown in Fig.  
113 S1.

### 114 ***2.4. Design of the electrochemical detector of paper based electrode***

115 To build the electrodes onto the patterned NC membrane, an additional pattern was drawn on a  
116 steel wafer with 1 mm thickness. Then the obtained pattern was cut by laser cutter. Resulted  
117 mask was placed on the working area of the NC membrane and carbon ink was applied to give  
118 shape as the working (WE) and counter electrode (CE). Additionally, we used Ag/AgCl ink to  
119 create the reference electrode (RE). Conductive pads were created by using copper wire. Before  
120 attaching the copper wire, wax printed NC membrane with electrodes was backed at 100 °C for  
121 5 min to let carbon ink fix on the NC membrane surface and also the wax melt and interpenetrate  
122 through the NC membrane. The electrode design is schematized in Fig. S2. The storage stability  
123 of the paper electrodes was evaluated and the results are given in Fig. S3.

124 ***2.5.Preparation of AuNPs modified electrodes***

125 AuNPs were deposited onto PE using chronoamperometry in 15 mM HAuCl<sub>4</sub> gold precursor  
126 aqueous solution. 5 μL of HAuCl<sub>4</sub> solution was dropped onto the PE and -0.3 V constant  
127 potential is applied during 600 s.

128 ***2.6.Probe immobilization on AuNPs modified electrodes***

129 5 μL of thiol liked miRNA-155 DNA probe is dropped and allowed to immobilize onto the  
130 AuNP-PE during 10 min. Then, washed with PBS (pH 7.40).

131 ***2.7.Hybridization of probe and miRNA-155***

132 5 μL of miRNA-155 target in various concentrations were applied to the electrode and washed  
133 with PBS (pH 7.40) after 5 min of incubation at room temperature.

134 ***2.8.Impedimetric measurement***

135 20 μL of 0.1 M KCl solution containing 5 mM [Fe(CN)<sub>6</sub>]<sup>3-/4-</sup> was dropped into the paper  
136 electrochemical cell through the channel.

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150 **3. Results and Discussion**

151 ***3.1. Microscopic and electrochemical characterization of modified/unmodified***  
152 ***electrodes***

153 Scanning electron microscopy (SEM) was used to characterize AuNPs modified paper based  
154 electrode. AuNPs were deposited on the working electrode surface by electrochemical  
155 reduction. While Fig. 1a,b show bare carbon paste PE at different magnification levels, Fig.  
156 1c,d show AuNPs deposited on PE surface. AuNPs were homogeneously distributed on the PE  
157 surface as shown in Fig. 1c,d. The average diameter of AuNPs was calculated as  $262 \pm 30$  nm.

158  
159 **Figure 1**

160  
161 In order to explore the electrochemical behavior of PE and AuNPs modified PE, cyclic  
162 voltammetry was performed. The anodic and cathodic currents;  $I_a$ ,  $I_c$ , relative charge;  $Q$  (C),  
163 and the surface areas of the PE and AuNPs modified PE are shown in Table S1. The  $I_c$  and  $I_a$   
164 of AuNPs modified PE (Fig. 2-I) were much larger than  $I_c$  and  $I_a$  of PE (Fig. 2-I). These results  
165 confirmed that the accelerated electron transfer occurred by means of AuNPs [38]. The  
166 electroactive surface areas (A) of PE and AuNPs-PE were calculated [39] as  $0.019 \text{ cm}^2$  and  
167  $0.037 \text{ cm}^2$  respectively. In comparison to the calculated surface area of PE, an increase about  
168 91 % at surface area of AuNP-PE was obtained by means of conductive behavior of AuNPs  
169 [38].

170  
171 **Figure 2.**

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173 EIS was also performed in order to obtain detailed information regarding modification process  
174 since measurements carried out in the absence/presence of AuNP modification (Fig. 2-II). The  
175 change in the value of  $R_{ct}$  was associated with the modification processes of PE surface. The  
176 measured  $R_{ct}$  of unmodified PE was  $7122.75 \pm 440$  Ohm (RSD %, 6.18 %,  $n=4$ ). After  
177 modification of AuNP, the  $R_{ct}$  exhibited a substantial decrease (161 folds) and measured as  
178  $44.12 \pm 3.76$  Ohm (RSD %, 8.53 %,  $n=4$ ). This observation indicates that the AuNPs with high

179 conductivity acted as a conductive layer for the charge transfer. These results are in good  
180 consistent with the CV results presented in this present study.

### 181 *Analytical performance*

182 The analytical performance of AuNP-PEs is tested through the detection of miRNA-155 target  
183 at different concentrations. Shown in Fig. 3, with the increase of miRNA-155 target  
184 concentration, the  $R_{ct}$  value increases over a wide concentration range of 0 – 1.5  $\mu\text{g/mL}$  and  
185 then decrease in the presence of 2  $\mu\text{g/mL}$  miRNA-155 target as shown in Fig. S4. The linear  
186 regression equation was  $R_{ct} (\text{Ohm}) = 16.57 [\text{miRNA-155}] (\mu\text{g/mL}) + 8.02$  with a correlation  
187 coefficient of 0.98 and the limit of detection (LOD) was calculated as 0.25  $\mu\text{g/mL}$  (33.8 nM)  
188 [40].

### 189 **Figure 3.**

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### 191 *3.2.Selectivity of the AuNPs modified electrodes*

192 To evaluate the specificity of the AuNP-PE, the interference effect of the noncomplementary  
193 and single base mismatched strands were also investigated. The efficiency of hybridization (HE  
194 %) is used as the evidence of the hybridization effectiveness [41] and the average  $R_{ct}$  values  
195 with the HE % can be seen in Table S2. HE % is accepted as 100 % for hybridization between  
196 probe and miRNA 155. The results clearly reveal that the AuNP-PE showed a sensitive  
197 behavior to its complementary target.

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### 199 **Figure 4.**

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### 201 *3.3.Analytical performance in serum media*

202 In the present study, the analytical performance of AuNP-PEs was tested in serum media (Fig.  
203 5). When the hybridization was performed in case of the increasing concentration of miRNA-  
204 155 target, the  $R_{ct}$  value increased among the concentration range of 0 – 4  $\mu\text{g/mL}$ , then  
205 decreased in the presence of 6  $\mu\text{g/mL}$  miRNA-155 target (Fig. S5). The linear regression  
206 equation was  $R_{ct} (\text{Ohm}) = 11.15 [\text{miRNA-155}] (\mu\text{g/mL}) + 36$  with  $R^2=0.98$  and the LOD was  
207 calculated as 0.69  $\mu\text{g/mL}$  (93.4 nM).



208 **Figure 5.**

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210 ***3.4.Selectivity in serum media***

211 The specificity of the AuNP-PE in serum media was then tested (Fig. S6). The HE %s were  
212 calculated as 13 % and 16 %, in case of possible hybridization/interaction between probe and  
213 NC, MM, respectively. On the other hand, HE %s were 65 % and 41 %, respectively after  
214 hybridization of probe and miR-155 target in the presence of NC or MM (Table S3).

215 According to our results related to the selectivity test in buffer and serum media, it can be said  
216 that the proposed AuNP-PEs exhibited more selective behavior in complex serum medium than  
217 buffer. These results showed that AuNP-PEs are able to perform microRNA detection in a  
218 sensitive and selective way even in a complex medium as serum.

219 Storage stability of the paper electrodes was evaluated. Fig. S3 shows the stability of the  
220 electrodes during 60 days of storage in dark conditions and without other special care.

221 Several reports related to the electrochemical detection of miRNAs were summarized in Table  
222 S4 [29,41-61]. In the present work, the impedimetric miRNA-155 analysis was accomplished  
223 in relatively shorter time (i.e 15 min) compared to earlier works shown in Table S4. The  
224 detection limit was found respectively to be 33.8 nM in PBS and 93.4 nM in FBS:PBS (1:1)  
225 diluted solution, which was comparable to earlier works [42-48] (Table S4).

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235 **4. Conclusion**

236 The gold nanoparticles (AuNPs) modified paper based electrode assemble system (AuNP-PE)  
237 was designed for the first time in this present study, and successfully applied to perform fast,  
238 sensitive, selective and quantitative miRNA-155 detection. Our results showed that the stability  
239 of these electrodes during 60 days of storage in dark conditions and without other special care.

240 Since this protocol exhibits more selective behavior in complex serum medium than buffer, it  
241 is obvious that AuNP-PEs could be able to perform selectively nucleic acid detection including  
242 miRNA, SNP, etc. even in a complex medium as serum.

243 AuNPs-PEs can be assesable as a promising platform for analysis in real serum samples for  
244 early diagnosis of cancer. It is important to mention that usage of a paper based microfluidic  
245 chip, except for isolation of target analyte from solution, provides us to benefit from the  
246 advantages of miniaturized systems comparison to the conventional batch technique.

247

248 **Conflicts of interest**

249 There are no conflicts of interest to declare

250

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459 **The list of figure captions:**

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461 **Figure 1.** SEM images of (a,b) unmodified paper electrode and (c,d) AuNPs modified paper  
462 electrode at magnification of 20000× and 50000x.

463 **Figure 2.** The concept of AuNPs modified paper electrode sensing system. Left, the sketch of  
464 the paper electrode sensing system. AuNP deposition is performed by chronoamperometry in  
465 15 mM HAuCl<sub>4</sub> aqueous solution by applying -0.3 V constant potential during 600 s. **(I)** CVs  
466 of PE and AuNP-PE. Supporting electrolyte solution is 0.1 M KCl containing 50 mM  
467 [Fe(CN)<sub>6</sub>]<sup>3-</sup>. **(II)** Nyquist diagrams of PE and AuNP-PE. Supporting electrolyte solution is 0.1  
468 M KCl containing 5 mM [Fe(CN)<sub>6</sub>]<sup>3-/4-</sup>.

469 **Figure 3.** **(A)** Nyquist diagrams obtained before / after hybridization of probe with 0.5, 1, 1.5,  
470 2 µg/mL miRNA 155 target. **(B)** The calibration graph obtained after hybridization between 0.5  
471 µg/mL miRNA 155 DNA probe and miRNA 155 target with its various concentrations from 0  
472 to 1.5 µg/mL (n=3).

473 **Figure 4.** Selectivity of AuNP-PE. Histograms representing the average R<sub>ct</sub> values obtained by  
474 (a) miRNA 155 probe/AuNP-PE, hybridization between probe and (b) miRNA 155, (c) non-  
475 complementary (NC), (d) single-base mismatched strand (MM), and mixture samples with (e)  
476 miRNA 155 and NC or (f) miRNA 155 and MM strand (n=3).

477 **Figure 5.** **(A)** Nyquist diagrams obtained before / after hybridization of probe with 2, 4, 6  
478 µg/mL miRNA 155 in 1:400 diluted FBS medium. **(B)** The calibration graph based on average  
479 R<sub>ct</sub> values obtained after hybridization between probe and miRNA 155 with its various  
480 concentrations from 0 to 4 µg/mL in 1:400 diluted FBS medium (n=3).

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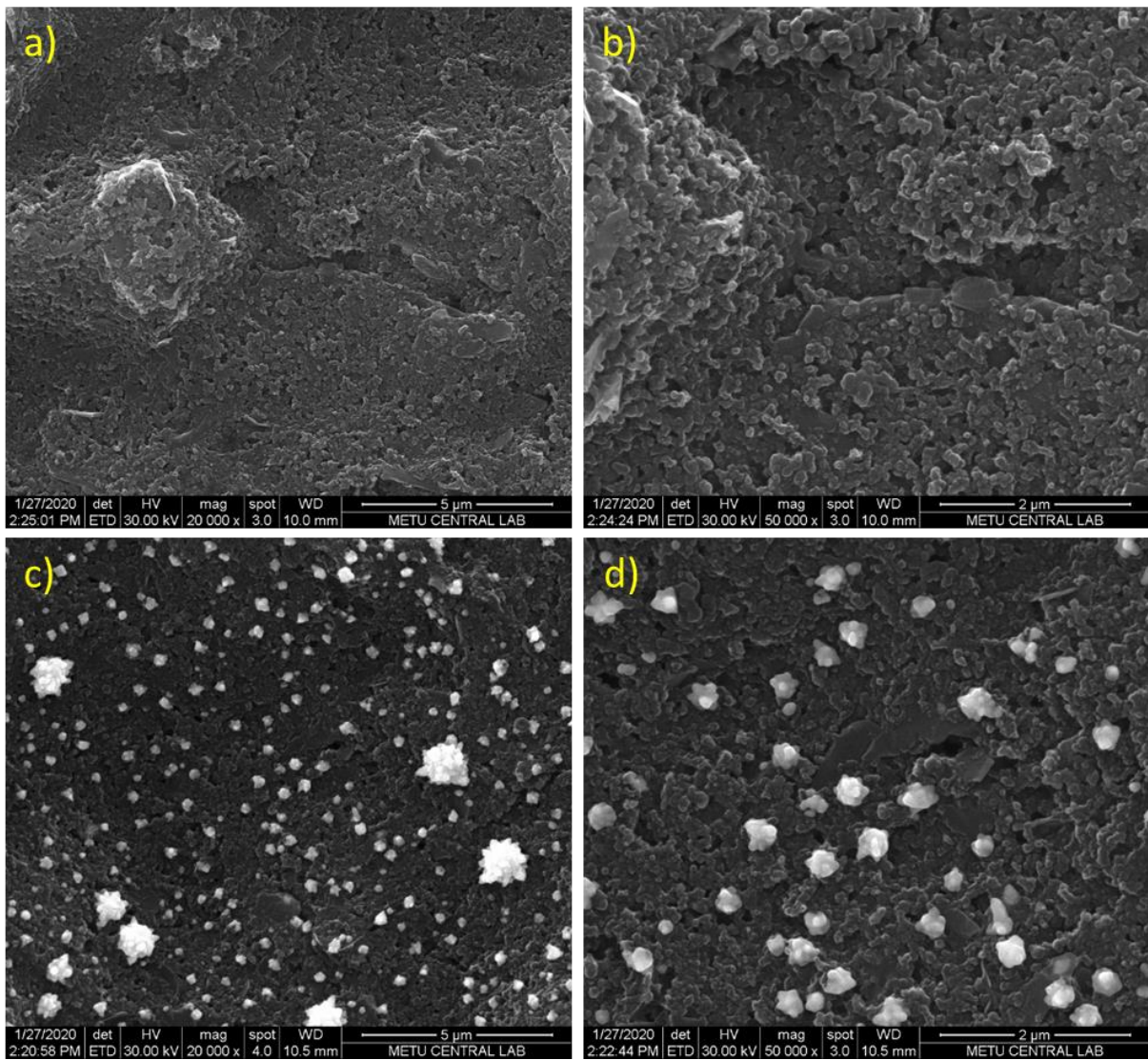
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# Figures

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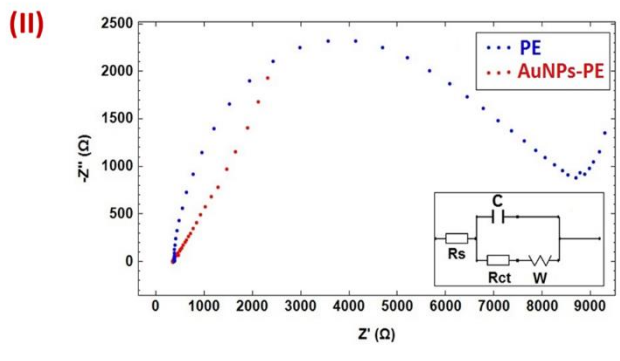
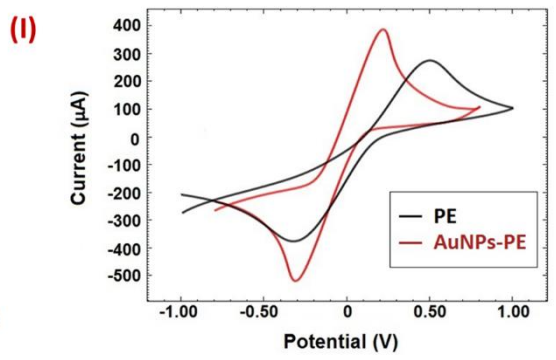
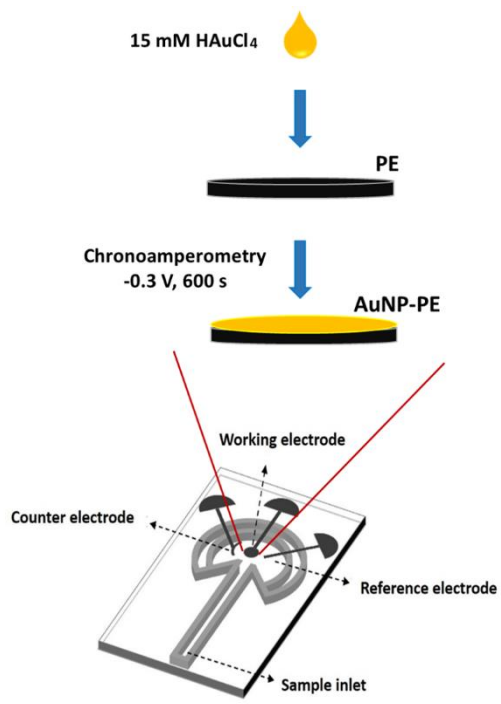


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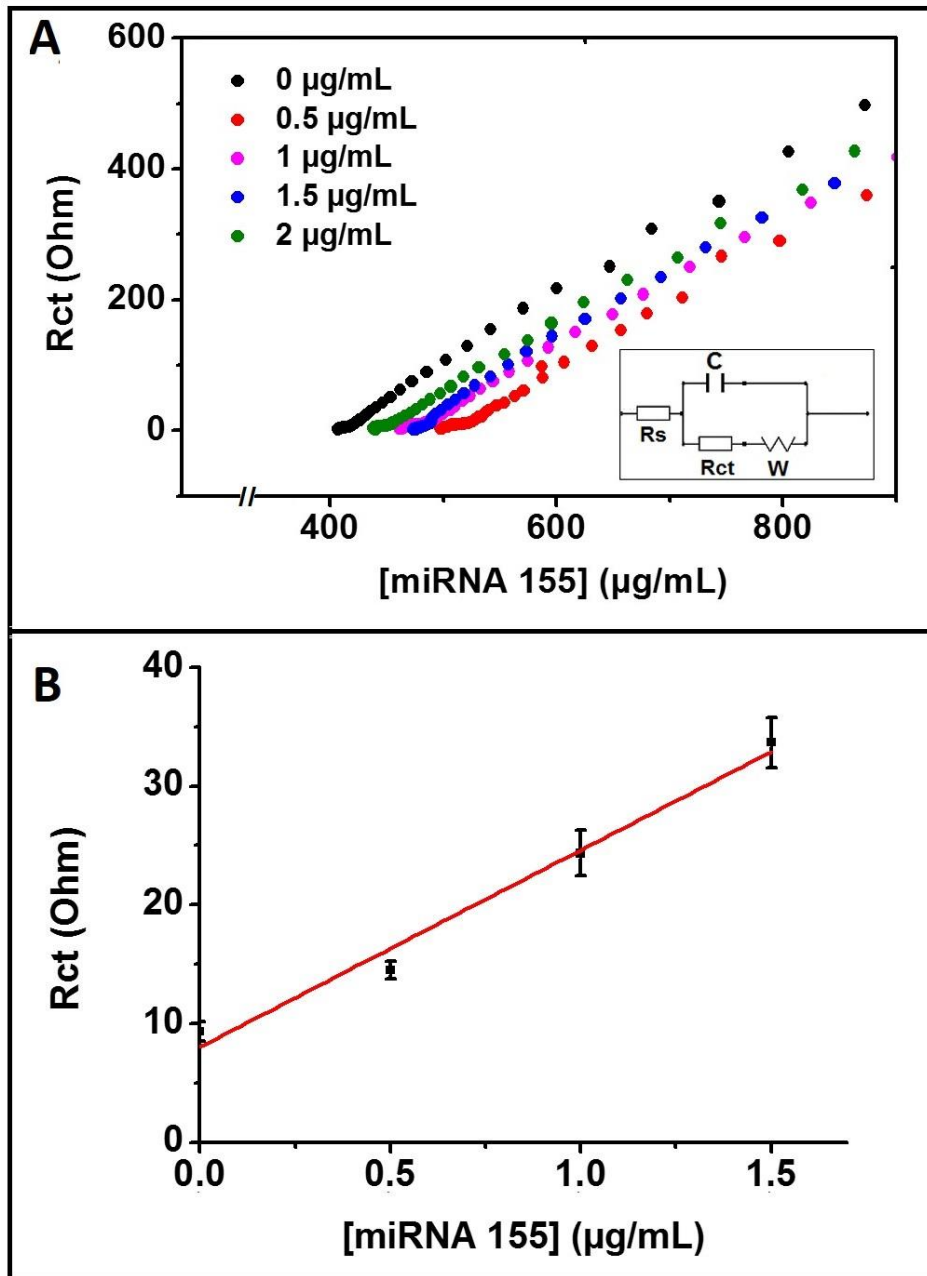
Figure 1.

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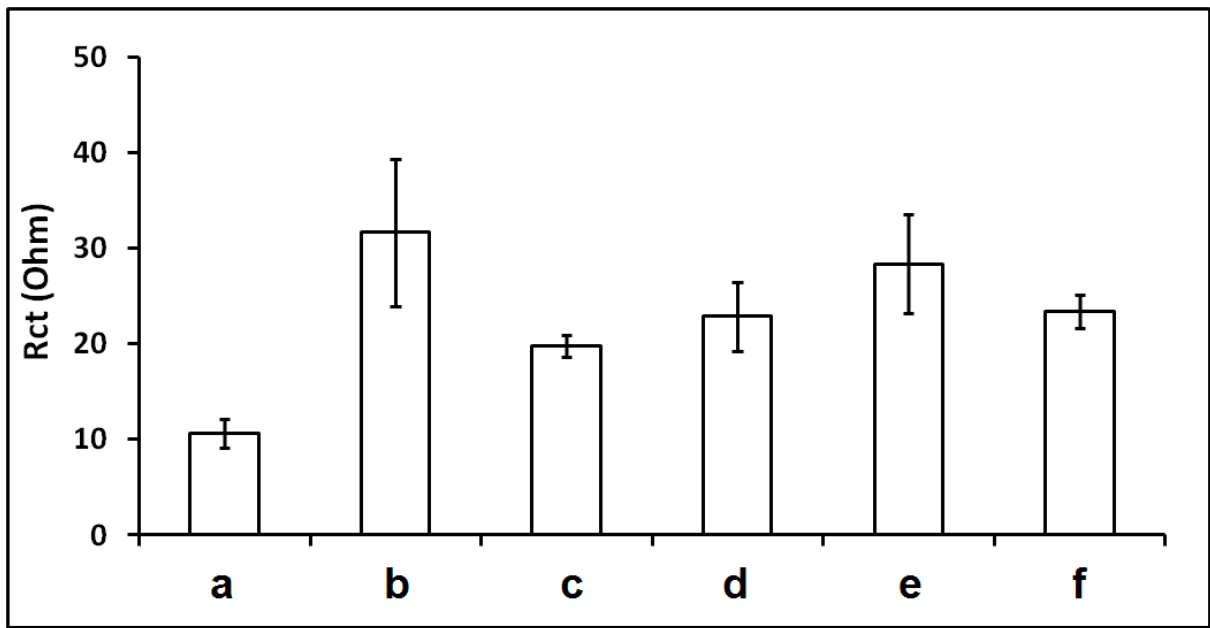
Figure 2.



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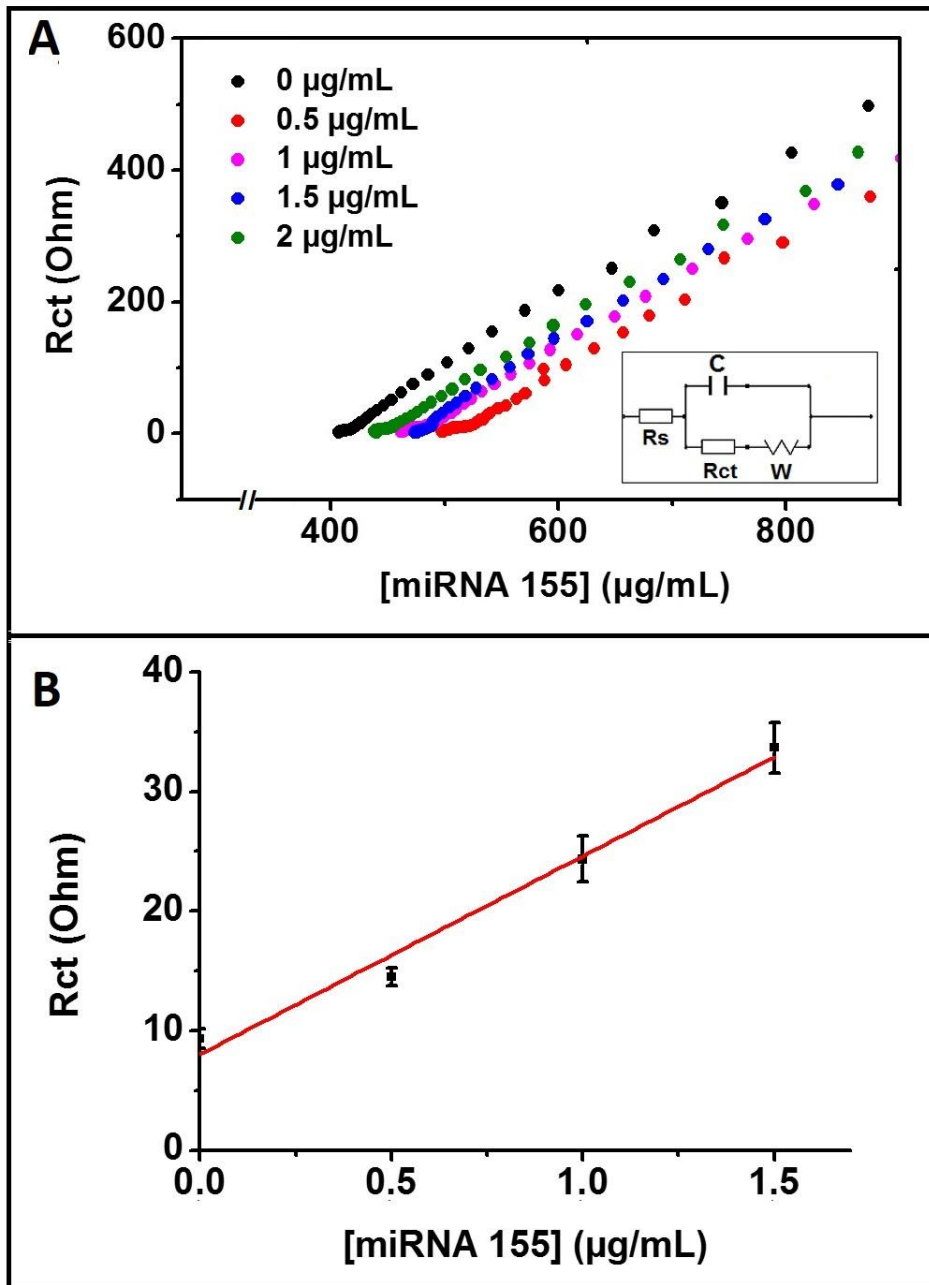
Figure 3.



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Figure 4.



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Figure 5.