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Author

Islam, Nazmul, Gorgannezhad, Lena, Masud, Mostafa Kamal, Tanaka, Shunsuke, Hossain, Md. Shahriar A., Yamauchi, Yusuke, Nguyen, Nam-Trung, Shiddiky, Muhammad

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Supporting Information

Graphene oxide-loaded iron oxide superparamagnetic nanoparticles for ultrasensitive electrocatalytic detection of microRNA

Md. Nazmul Islam,[a,b] Lena Gorgannezhad,[a,b] Mostafa Kamal Masud,[b,c] Shunsuke Tanaka, [c] Md. Shahriar Al Hossain,[c] Yusuke Yamauchi,[c,d,e] Nam-Trung Nguyen,[b] and Muhammad J.A. Shiddiky*[a,b]

[a] Dr M. N. Islam, L. Gorgannezhad, Dr. M. J. A. Shiddiky

School of Environment and Science, Griffith University

Nathan Campus, QLD 4111 (Australia) E-mail: m.shiddiky@griffith.edu.au

[b] Dr M. N. Islam, L. Gorgannezhad, M. K. Masud, Prof. N.-T. Nguyen, Dr. M. J. A. Shiddiky

Queensland Micro- and Nanotechnology Centre, Griffith University

Nathan Campus, QLD 4111 (Australia)

[c] M. K. Masud, S. Tanaka, Dr. M. S. A. Hossain, Prof. Y. Yamauchi

Australian Institute for Innovative Materials (AIIM), University of Wollongong, Squires Way, Innovation Campus, North Wollongong, NSW 2500 (Australia)

[d] Prof. Y. Yamauchi

School of Chemical Engineering & Australian Institute for Bioengineering and Nanotechnology (AIBN), The University of Queensland, Brisbane, QLD 4072 (Australia)

[e] Prof. Y. Yamauchi

International Center for Materials Nanoarchitectonics (MANA), National Institute for Materials Science (NIMS), 1-1 Namiki, Tsukuba, Ibaraki 305-0044 (Japan)

Experimental Section

Reagents and Instrumentations. All the synthetic oligonucleotides were purchased from Integrated DNA Technologies (USA) (**Table S1**). Reagent grade hexaammineruthenium(III) chloride and phosphate buffered saline (PBS) tablet (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride) were purchased from Sigma-Aldrich (USA). Analytical grade hydrochloric acid (HCl) were obtained from Chem-Supply (Australia) and Tris from VWR Life Science (Australia). UltrapureTM DNase/RNase-free distilled water (Invitrogen, Australia) was used. All the electrochemical measurements were performed with a CHI650 electrochemical workstation (CH Instrument, USA). Cyclic voltammetry (CV) and chronoamperometry experiments were done in a 3-mL volume single-compartment cell with a conventional three-electrode system (glassy-carbon working (GCE), platinum auxiliary, and Ag/AgCl reference electrodes, CH Instrument, USA). Chronocoulometry (CC) measurements were carried out on a screen-printed carbon electrode (SPCE) with a potential range between 0.0 and -500 mV with 250 ms pulse width and 2.0 ms sample interval. SPCE was printed on a ceramic substrate (length 33 × width 10 × height 0.5 mm) (DRP-150) and purchased from Dropsens (Spain). In the three-electrode system of SPCE, working (4 mm diameter) and counter electrode are made of carbon and platinum respectively, the reference electrode and electric contacts are made of silver.

Synthesis of Graphene Oxide-loaded Iron Oxide Superparamagnetic Nanoparticles

Synthesis of GO sheets. Graphene oxide was synthesized by the modified Hummer's method. Sodium nitrate (0.3 g) was firstly dissolved in sulfuric acid solution (10 mL) under constant stirring. Nanographite platelet powder was added to the solution which was further stirred for 30 min. After subsequently adding $KMNO_4$ (0.30 g), the mixture was aged for 1 h. Finally, H_2O_2 (10 mL) was added to the mixture under constant stirring to obtain GO sheets.

Synthesis of Prussian blue (PB) nanoparticles. A 40 mL aqueous solution containing 3.24 g of $FeCl_3 \cdot 6H_2O$ and 3.24 g of TSCD was mixed with another 40 mL aqueous solution containing 4.36 g of $Na[Fe(CN)_6] \cdot 10H_2O$, and the mixture was vigorously stirred for 1 h before being statically aged overnight to ensure a complete reaction. Finally, the PB nanoparticles were obtained by centrifugation.

Synthesis of GO/IO hybrid materials. The above-prepared GO and PB suspension was diluted down to 2.0 $mg \cdot mL^{-1}$ by adding water before being mixed together under sonication with specific weight ratios of 25:75. The mixtures were continuously treated by sonication for 30 min, and then aged overnight. The GO/PB hybrid precipitates settling at the bottom of the vial were washed with water and ethanol several times, before being dried at room temperature. The GO/IO hybrids were obtained by calcining the GO/PB powders at 400 °C at a heating rate of 1.0 °C min^{-1}.

RNA Preparation. SKOV3 ovarian cancer and MeT- 5A non-cancerous cell lines were cultured in RPMI-1640 growth medium (Life Technologies, Australia) supplemented with 10% fetal bovine serum (Life Technologies, Australia) and 1% penicillin/streptomycin (Life Technologies, Australia) in a humidified incubator containing 5% CO_2 at 37 °C. The SKOV3 and MeT- 5A cells were collected after 4 and 7 days, respectively, for subsequent cell counting and RNA extraction. Total RNA was extracted following the standard protocol from *RNeasy Mini Kit* (Qiagen, Germany).

Probe Hybridization and Magnetic Purification of RNA. For probe hybridization, 10 µL of RNA sample was mixed with 10 µL of 5× SSC buffer (pH 7.0) and 10 µL of 10 µM biotinylated capture probes. The mixture solution was heated at 65 °C for 2.0 min and placed on a thermomixer for 1.0 h at room temperature (25°C) to allow the capture probe hybridization to target miRNA. Next, 20 µL of streptavidin-labeled (MyOne Streptavidin C1, Invitrogen) magnetic beads were washed with 2× washing and binding (B&W) buffer (10 mM Tris-HCl,

pH 7.5; 1.0 mM EDTA; 2.0 M NaCl) and resuspended in 20 µL of 2× B&W buffer. The preparation was then added to biotinylated capture probes- miRNA complex. The resultant solution was incubated for 30 min at room temperature to allow the formation of dynabead-functionalized target miRNA complex. The magnetic beads bound miRNA complex was separated using a magnet, washed thrice with 2× B&W buffer, and resuspended in 10 µL of RNase-free water. The magnetically captured isolates were heated for 2 min at 95 °C, and the heat-released miRNAs were immediately collected from the supernatant using an external magnet. Then, 5.0 µL of the released miRNA was diluted with 15 µL of 5× SSC buffer (pH 7.0) for electrochemical readout.

Evaluation of Electrocatalytic Activity of GO/IO Hybrid Materials. A GCE was polished using 0.3 and 0.05 mm alumina slurry (CH Instrument, Inc. USA) followed by an adequate wash with water. After sonication with nitric acid and water, the electrode was washed thoroughly, and dried at room temperature. To assess the electrocatalytic activity of GO/IO hybrids, 4.0 µg of a colloidal suspension of GO/IO hybrid materials were drop-dried onto the working surface of the GCE electrode. The electrocatalytic activity GO/IO hybrids towards the reduction of RuHex was studied using cyclic voltammetry with the conventional three-electrode system where the working surface of GCE electrode was modified with GO/IO hybrid materials. The chronoamperometric readout was obtained at -0.25V versus Ag/AgCl in optimum condition. The current response due to the successive addition of different concertations (10 – 1100 µM) of RuHex was monitored. The apparent Michaelis-Menten constant (K_m^{app}) of GCE/ GO-IO hybrids was determined from the Michaelis-Menten equation which is as follows.[1]

$$I = \frac{I_{\max}[S]}{K_m^{app} + [S]}$$

where I is the steady-state current, I_{max} is the maximum current measured under the condition of catalyst saturation, [S] is the substrate concentration, and K_m^{app} (i.e., the substrate concentration needed to reach the half of I_{max}) is the Michaelis- Menten constant used to indicate the affinity of the catalyst towards the substrate. The electrochemical version of Lineweaver–Burk equation [2] was also obtained with the rearrangement of Michaelis-Menten equation which is as follows

$$\frac{1}{I} = \frac{K_m{}^{app}}{I_{\max}} \frac{1}{[S]} + \frac{1}{I_{\max}}$$

Electrochemical Detection. The effective areas of SPCE was determined by the measurement of the peak current obtained as a function of scan rate under cyclic voltammetric conditions for the one-electron reduction of $[Fe(CN)_6]^{3-}$ [2.0 mM $K_3Fe(CN)_6$ in 10 mM PBS (0.5 M KCl)] using the Randles- Sevcik equation, as shown before.[3] The chronocoulometry (CC) was performed in 40 mM Tris buffer (pH 7.4) in the absence and presence of 50 µM RuHex with the potential step of 500 mV with the 250 ms pulse width and 2 ms sample interval. For detecting miRNA, SPCE was modified with 4.0 µL of GO/IO hybrid materials (1.0 µg/µL) and magnetically bound on a permanent magnet followed by a PBS wash to remove any unattached or loosely attached nanoparticles on the electrode surface. 4.0 µL of magnetically purified miRNA (diluted in 5× SSC buffer) sample was then directly put onto the GO/IO hybrids modified SPCE surface and incubated for 30 min followed by PBS washing. The electrode was then incubated with 10 µL of 50 µM RuHex so that positively charged Ru^{3+} can bind with the negatively charged phosphate backbone of adsorbed miRNA. After PBS wash, the CC charge derived from the surface-confined RuHex bound with miRNA was measured in 40 mM Tris buffer (pH 7.4). We further measured the CC using 4.0 mM $[Fe(CN)_6]^{3-}$ electrolyte prepared in 40 mM Tris-HCl buffer (pH 7.4). To calculate the number of cationic redox molecules

electrostatically associated with the surface-attached anionic phosphate backbone of miRNA, following integrated Cottrell equation was used.[4]

$$Q = \frac{2nFAD_0^{1/2}C_0^*}{\pi^{1/2}} t^{1/2} + Q_{dl} + nFA\Gamma_0$$

Here the total charge is Q at a time 't', n is the number of electrons involved in electrode reaction, F is Faraday constant (C/equivalent), A is the electrode area (cm^2), D_o is the diffusion coefficient (cm^2/s), C_o^* is the bulk concentration (mol/cm^2), Γ_0 is represent the amount of RuHex-confined near electrode surface and nFAΓ_0 (known as surface excess) is the charge obtained by adsorbed miRNA. CC curves were constructed by plotting the charge versus square-root of time ($t^{1/2}/s^{-1/2}$) in the presence and absence RuHex. Q and Q_{dl} were estimated from the intercept of these two curves at t = 0 where Q represents the total charge comprising both Faradic and non-Faradic (capacitive) charges. Hence, the corresponding charge of RuHex deriving from electrostatically bound to surface confined RNA (Q_{RNA}) can be calculated by subtracting the Q_{dl} as follows

$$Q_{RNA} = Q - Q_{dl}$$

Table

Table S1. Oligonucleotide Sequences

Oligos	5’-Sequences-3’
miR-21 capture probe	TGACCGACCCAGTGAGGAAGTTTTCTCT/3Bio/
Synthetic miR-21	ArGrArGrArArArArCrUrUrCrArCrUrGrGrGrUrCrGrGrUrCrA
Synthetic miR-107	ArGrCrArGrCrArUrUrGrUrArCrArGrGrGrCrUrArUrrCrA
Synthetic miRNA-338-3p	UrCrCrArGrCrArUrCrArGrUrGrArUrUrUrUrGrUrUrG

Supporting Figures

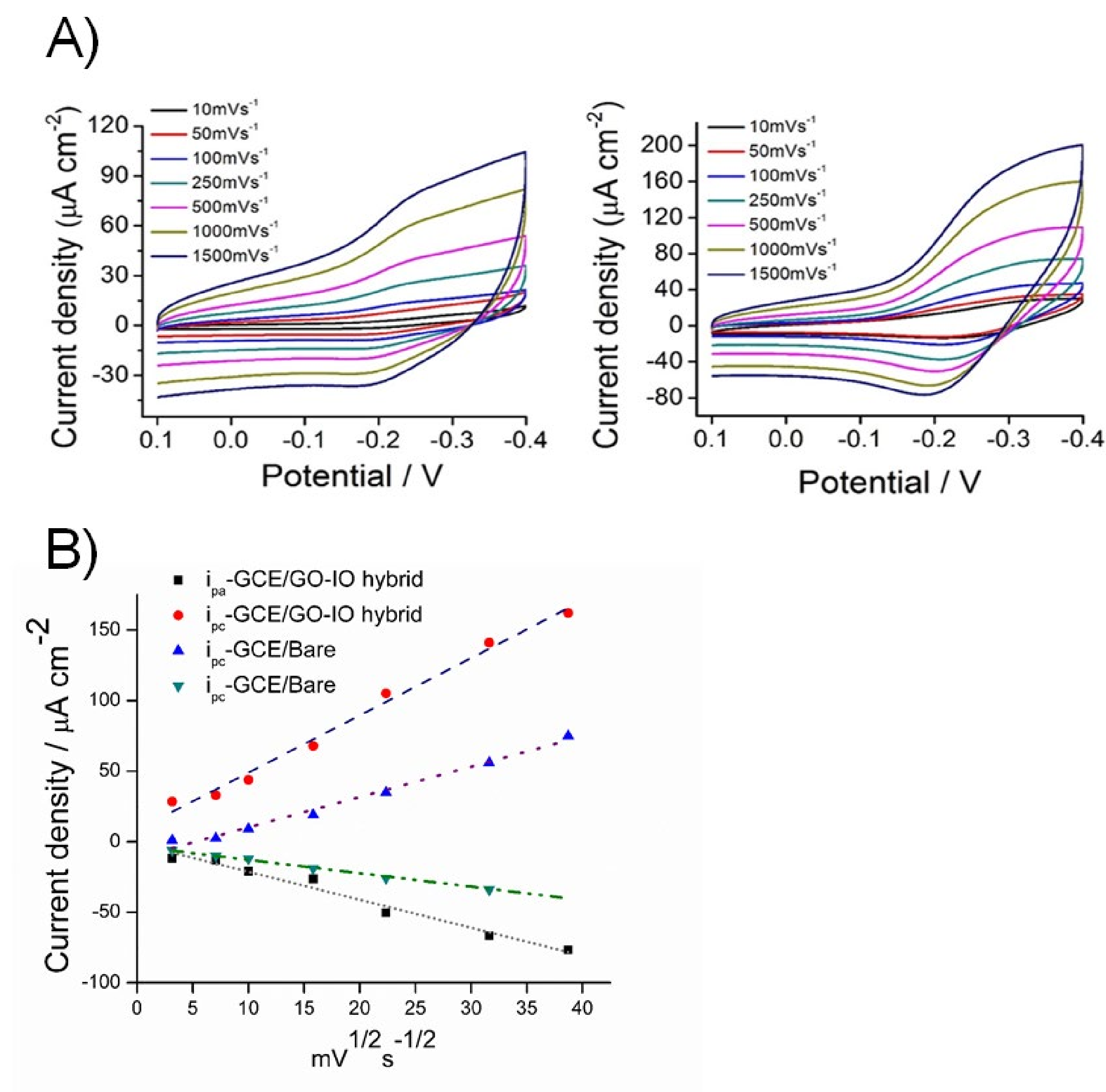

Figure S1. (A) Typical cyclic voltammograms obtained at unmodified-GCE (top, left) and GO/IO hybrid modified- GCE (top, right) electrodes at different scan rate (50 µM RuHex, 0.01 M PBS, pH 7.0). (B) Corresponding curves for i_{pc} and i_{pa} (current density) as a function of $v^{1/2}$.

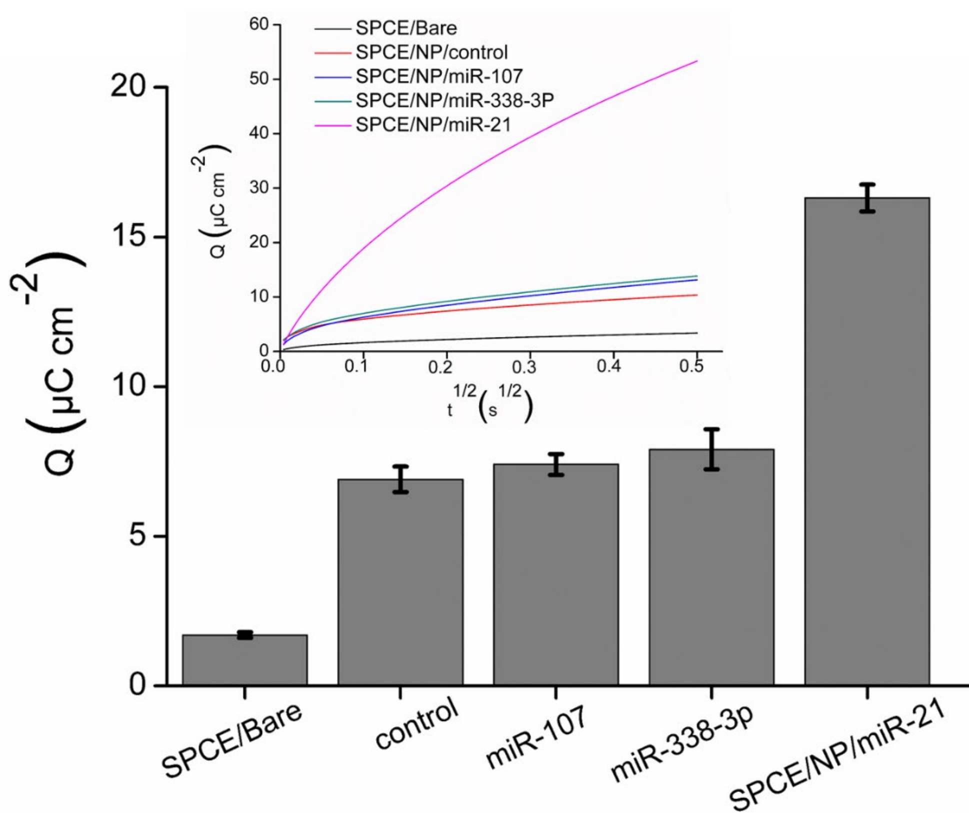

Figure S2. Assay specificity without an electrocatalytic cycle. Corresponding charge density data for the SPCE/Bare, control, non-complementary miR-107 and miR-338-3p, target miR-21; inset, corresponding CC curves (Q vs $t^{1/2}$). Each data point represents the average of three independent trials, and error bars represent the standard deviation of measurements (% RSD = <5%, for n = 3).

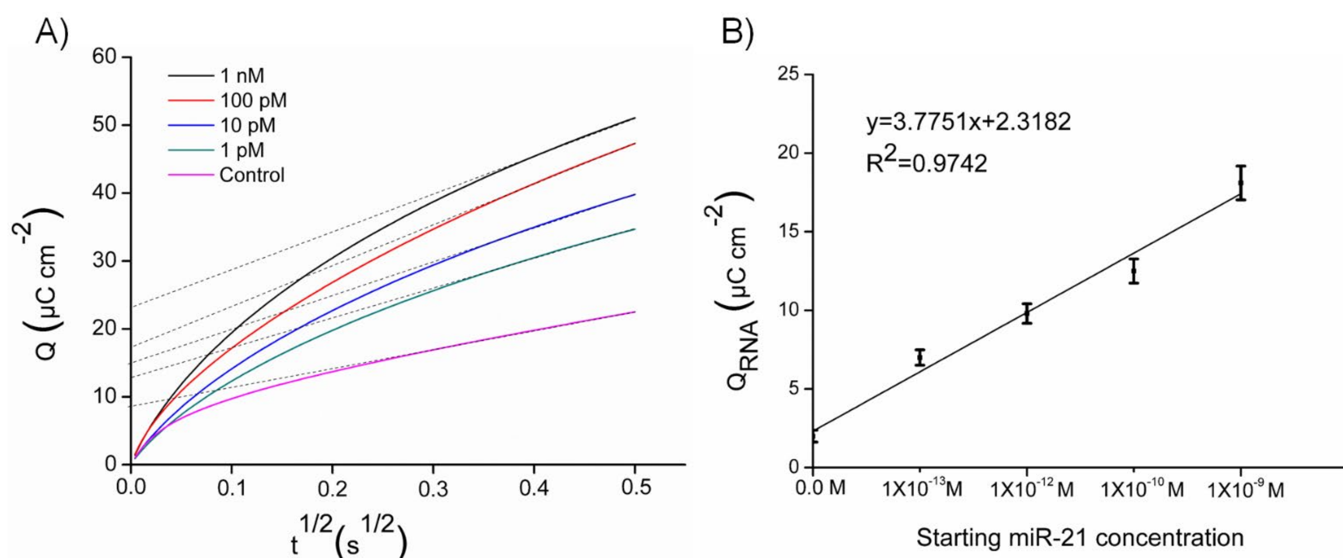

Figure S3. Sensitivity without electrocatalytic cycle (A) Typical CC curves (Q vs. $t^{1/2}$) for the SPCE/control and 1.0 pM- 1.0 nM of miR-21. (B) Corresponding calibration plot of Q_{RNA} - concentration profile across the range of 1.0 pM- 1.0 nM miR-21. Each data point represents the average of three independent trials, and error bars represent the standard deviation of measurements (% RSD = <5%, for n = 3).

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