Supplementary Material (ESI) for Chemical Communications

Electronic Supporting Information

for

A Highly Sensitive MicroRNA Biosensor Based on Ruthenium Oxide Nanoparticle-Initiated Polymerization of Aniline

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Reagents. Thiolated PNA CPs used in this work were custom-made by Eurogentec (Herstal, Belgium) and all other oligonucleotides of PCR purity were from 1st Base Pte Ltd (Singapore). Total RNA extraction cocktail TRIzol reagent was from Invitrogen (Invitrogen, Carlsbad, CA). Aniline (99.5%), 4-(2-aminoethyl) pyridine (AEP), and 4-mercaptoaniline (MAn, 97%) were from Sigma-Aldrich (St Louis, MO). $RuCl_3 \cdot xH_2O$(~40% Ru) was purchased from Merck. All other reagents of certified analytical grade were obtained from Sigma-Aldrich and used without further purification. A pH 8.5 10 mM Tris-HCl-1.0 mM EDTA-0.10 M NaCl (TE) buffer solution was used as the hybridization and washing buffer. A pH 4.0 0.10 M acetate buffer was used as the deposition buffer for polyaniline (PAn) as well as the supporting electrolyte for SWV quantification.

Apparatus. Electrochemical experiments were carried out using a CH Instruments Model 660A electrochemical workstation coupled with a low current module in a Faraday cage (CH Instruments, Austin, TX). A conventional three-electrode system, consisting of a 3.0-mm-diameter gold working electrode, a Ag/AgCl reference electrode (Bioanalytical Systems, West Lafayette, IN, USA), and a platinum wire counter electrode, was used in all electrochemical measurements. To avoid the spreading of the sample droplet beyond the 3.0-mm diameter

working area, a patterned hydrophobic film was applied to the gold electrode after CP immobilization. The electrochemical techniques used are cyclic voltammetry and square wave voltammetry (SWV). SWV experiments were conducted with an amplitude of 50 mV, a step of 1.0 mV, and a frequency of 200 Hz. Transmission electron microscopic (TEM) experiments were performed on a FEI Tecnai G2 F20 electron microscope operated at 200 kV. UV-visible and fluorescence spectra were recorded on a V-570 UV/VIS/NIR spectrophotometer (JASCO Corp., Japan).

Synthesis and functionalization of RuO_2 nanoparticles (NPs). The synthesis and functionalization of the RuO_2 NPs is as follows: To a magnetically stirred solution of 0.10 g of $RuCl_3$ in 25 mL of methanol was slowly added 10 ml of 20 mg/mL NaOH in methanol. The solution was heated to 50 ºC for 30 min with continuous stirring. 25 mL of water was then added and the reaction mixture was transferred to a 200-mL Teflon-lined stainless-steel autoclave and annealed at 150–180°C for a certain period of time. The RuO_2 NPs were collected and purified by multiple centrifugation-decantation-dispersion cycles with methanol and were dried overnight at 100°C in vacuum. Next, AEP dissolved in water, was added to the RuO_2 NP suspension to a final concentration of 1.0 mmol/L under stirring. 25 mL of ethanol was added after 60 min of stirring and the mixture was centrifuged at 12000 rpm. The RuO_2 NPs were then washed and centrifuged several times with 50% ethanol. To ensure a reasonably high catalytic activity while providing sufficient amine groups on their surfaces, the RuO_2 NPs should be moderately coated. Too high an AEP concentration would result in 'over-coating', and hence substantially diminishes the catalytic activity by as much as 60%.

MicroRNA extraction and tagging. Total RNA was extracted using the TRIzol reagent according to the manufacturer's recommended protocol. The total RNA purity and concentration were determined by UV-vis spectrophotometry.[1] MicroRNAs in the total RNA

were enriched using a Montage spin column YM-50 (Millipore Corporation). The copy number of a given miRNA in the total RNA was determined by Northern blot. For electrochemical detection, miRNA was tagged as following: To 5.0 µg of total RNA solution was added 20 µl of 5 mM sodium periodate. The oxidization of the 3' overhang of miRNA was carried out at 25°C in the dark for 60 min. Then, 2-fold excess of sodium sulfite over sodium periodate was added to the reaction mixture followed by a 10-min incubation at 25°C. Finally, 0.50 mg/mL of the AEP-coated RuO_2 NPs was added and the mixture was incubated at 37°C for 3 h. The miRNA was covalently linked to the RuO_2 NPs through the formation of an imine bond between aldehyde on the miRNA and amine on the RuO_2 NP. The tagged miRNAs were collected and purified by two centrifugation-decantation-dispersion cycles with water and stored in a -20°C freezer.

Biosensor preparation, hybridization, and detection. To fabricate the working electrode, a titanium adhesion layer of 30–50 Å was first electron-beam evaporated onto an optically polished p-type single-crystal silicon wafer (111). A 200-nm gold film was deposited in a SEC 1000-RAP electron beam evaporator (CHA Industries, Fremont, CA). Gold (99.99%) was evaporated from a tungsten boat at a pressure lower than 2×10^{-6} Torr, and a deposition rate of 0.1–0.2 nm/s. Following gold deposition, the silicon wafer was annealed at 250 °C in air for 3 h. Immediately before CP immobilization, the gold coated silicon substrate was thoroughly cleaned in a freshly-prepared Piranha solution (CAUTION: Piranha solution is a powerful oxidizing agent and reacts violently with organic compounds.) and copiously rinsed with Milli-Q water followed by a 10-min sonication in absolute ethanol. Initial CP adsorption was accomplished by immersing the gold substrate in a phosphate-buffered saline (PBS) solution of 100 µg/mL CP at room temperature. After the adsorption, the gold substrate was copiously rinsed with PBS and soaked in stirred PBS for 10 min, rinsed again, and blown dry with a stream of air, a procedure

aimed at removing any nonspecifically adsorbed materials. To further improve the quality and stability, the CP-coated gold substrate was immersed in 2.0 mg/mL MAn for 2 h. During this treatment, MAn molecules fill in the defects via strong interaction between thiol and gold, forming a mixed monolayer with the CPs. Excess MAn molecules were rinsed off, and the electrode was washed by immersion in stirred ethanol for 10 min, followed by a thorough rinsing with water. The surface density of the immobilized PNA CPs was found to be in the range of 5.0–8.0 pmol/cm^2.[2] A patterned 2-mm-thick, adhesive, spacing/insulating layer was assembled on the top of the slide, forming a low-density array of 20–30 3 mm-diameter individual electrodes. The hybridization of the sample miRNA and its electrochemical detection were carried out as follows: First, the biosensor was placed in a moisture saturated environmental chamber maintained at 50ºC. A 2.0-µl aliquot of hybridization solution containing the RuO_2 NP-tagged miRNA was uniformly spread onto the biosensor. It was then rinsed thoroughly with a blank hybridization buffer at 50ºC after a 60-min hybridization period. Finally, the biosensor was incubated in a mixture of aniline/H_2O_2 (50 mM/100 mM) in the acetate buffer for 60 min. The PAn oxidation current in SWV was measured in the pH 4.0 0.10 M acetate buffer. In the case of low miRNA concentrations, smoothing was applied after each measurement to remove random noise and electromagnetic interference. All potentials reported in this work were referred to the Ag/AgCl electrode and all experiments were carried out at room temperature, unless otherwise stated.

Formation of RuO_2 NPs and their catalytic activities. Figure S1 is a TEM micrograph of the RuO_2 NPs, showing nearly spherical particles with a diameter of 2–3 nm. XPS analysis indicated that no detectable Ru(III) was found and the NPs are indeed made of pure Ru(IV) (Figure S2). A series of diffraction rings of the (110), (101), (200), (111), and (211) planes in the selected-area electron diffraction (SAED) pattern (Figure S1 insert) suggested that the NPs are

essentially RuO_2 and polycrystalline in nature. Smaller RuO_2 NPs down to 1.0 nm can be prepared with a higher $RuCl_3$ concentration while keeping the Ru/NaOH ratio unchanged.

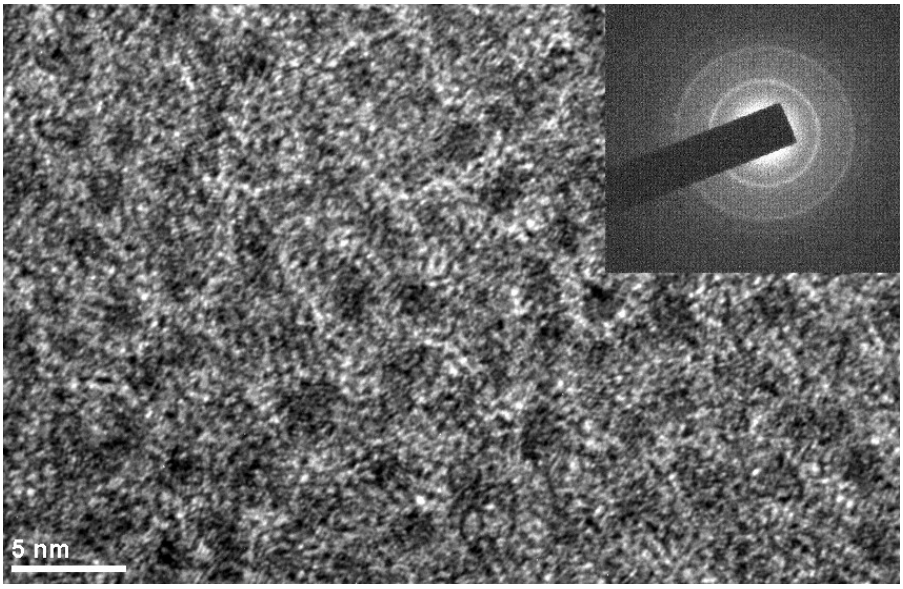

Fig. S1. TEM micrograph of RuO_2 NPs. Insert: SAED pattern of RuO_2 NPs.

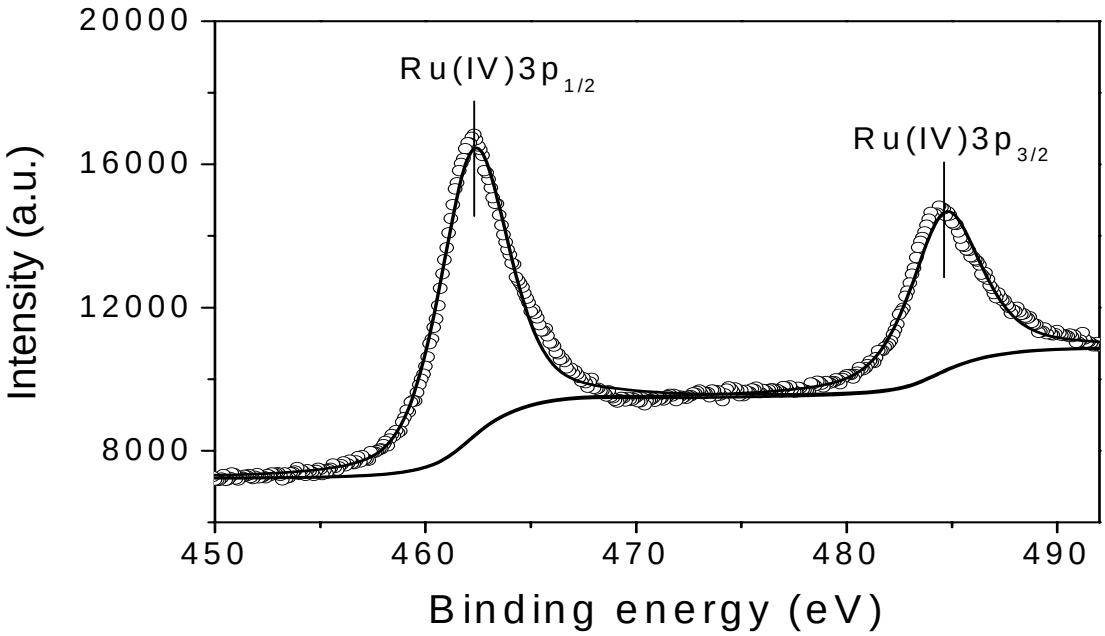

Fig. S2. XPS spectrum of RuO_2 NPs.

When a tiny amount of the RuO_2 NPs (<1.0 μg/mL) was added to the acetate buffer containing 20 mM aniline and 100 mM H_2O_2, a brown color developed slowly and followed

by the appearance of some brown precipitate. This initial observation prompted us to look into the detail of this catalytic activity and the potential use of the RuO_2 NPs as catalytic tags in biosensors. Figure S3 shows UV-vis absorption spectra of the mixture of aniline and H_2O_2 before and after the addition of the RuO_2 NPs. The spectrum of the aniline and H_2O_2 mixture before the addition of the RuO_2 NPs was practically featureless from 350 to 750 nm. On the contrary, two absorption peaks at 430 and 550 nm, corresponding to polaron/bipolaron transition[3] and the quinoid rings of the emeraldine base form of polyaniline,[4] respectively, slowly developed after the addition of the RuO_2 NPs. And brown precipitate was obtained after a prolonged period of incubation. This indicates that the RuO_2 NPs effectively catalyze the polymerization of aniline. Similar to the horseradish peroxidase-catalyzed polymerization of aniline,[5] nucleic acid strands, including miRNA strands, can serve as templates for the deposition of PAn. Consequently, PAn was exclusively grafted onto the miRNA strands.

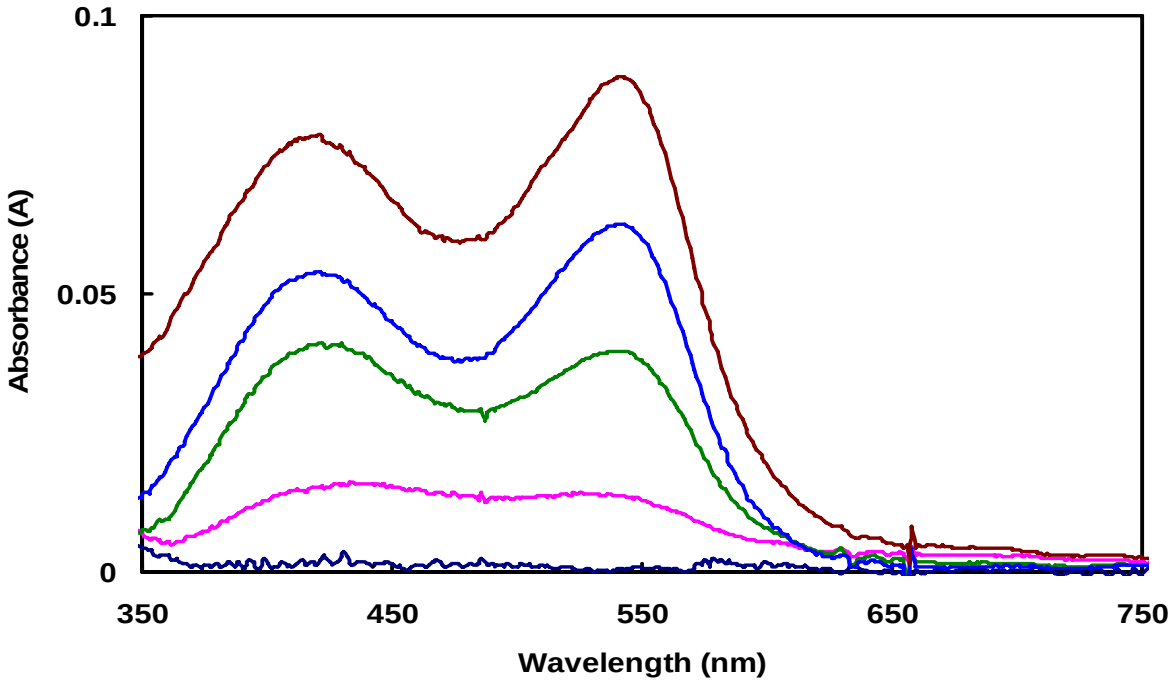

Fig. S3. UV-vis adsorption spectra of 20 mM aniline+100 mM H_2O_2 in pH 4.0 0.10 M acetate buffer. From bottom to top: 0, 15, 30, 60, and 90 min after the addition of the RuO_2 NPs.

Calibration curve. As shown in Figure 4S, the 60-min incubation period generated a dynamic range of 5.0 fM–2.0 pM with a relative standard derivation of <15% at 0.1 pM. The detection limit, defined as a signal/noise ratio of 3.0, was found to be ~2.0 fM.

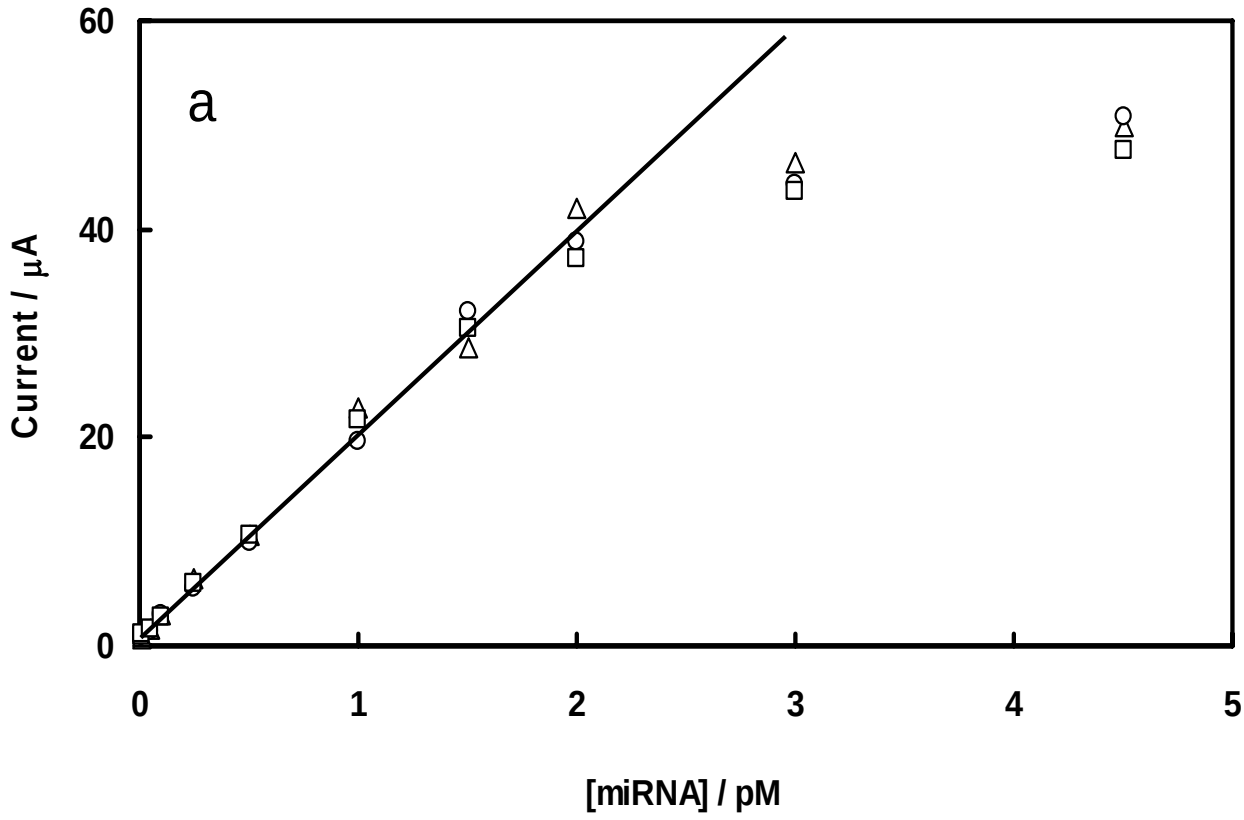

Fig. S4. Calibration curves for (o) miR-720, (∇) let-7c, and (□) miR-1248. 60 min incubation in 50 mM aniline/100 mM H_2O_2 in pH 4.0 0.10 M acetate buffer; supporting electrolyte pH 4.0 0.10 M acetate buffer, pulse amplitude of 50 mV, step 1.0 mV, and frequency 200 Hz.

MicroRNA expression analysis. Expression levels of three representative let-7 miRNAs of let-7 family (Table S1) were analyzed by the proposed biosensor and by Northern blot. As listed in Table S2, these results obtained with the biosensor are in good agreement with those of Northern blot. Compared to the copy numbers found in normal cells, all three miRNAs are down-regulated in cancer cells.

Table S1. Sequence information of let-7 family.[6]

MicroRNA	Sequence (5'→3')
let-7a	UGAGGUAGUAGGUUGUAUAGUU
let-7b	UGAGGUAGUAGGUUGUGUGGUU
let-7c	UGAGGUAGUAGGUUGUAUGGUU
let-7d	AGAGGUAGUAGGUUGCAUAGUU
let-7e	UGAGGUAGGAGGUUGUAUAGUU
let-7f	UGAGGUAGUAGAUUGUAUAGUU
let-7g	UGAGGUAGUAGUUUGUACAGUU
let-7i	UGAGGUAGUAGUUUGUGCUGUU
let-7c pre-miRNA	GCAUCCGGGUUGAGGUAGUAGGUUGUAUGGUUUAGAGUUACAC CCUGGGAGUUAACUGUACAACCUUCUAGCUUUCCUUGGAGC

Table S2. Analysis of miRNA in total RNA extracted from cell lines

	let-7a (10^7 **copy/μg RNA)**	**Let-7b** (10^7 **copy/μg RNA)**	**Let-7c** (10^7**copy/μg RNA)**
HeLa Cells	1.82±0.20	2.45±0.29	3.90±0.45
	(1.73±0.15)*	(2.29±0.21)	(3.81±0.32)
Lung Cancer Cells	1.05±0.12	2.73±0.32	2.11±0.24
	(1.1±0.10)	(2.81±0.35)	(2.17±0.23)
Normal Cells	4.68±0.55	8.32±0.90	6.95±0.82
	(4.55±0.39)	(8.55±0.88)	(6.02±0.74)

* Data in brackets were obtained by Northern blot.

References

1 J. Sambrook, E. F. Fritsch and T. Maniatis, *Molecular Cloning, A Laboratory Manual, 2nd ed,* Cold Spring Harbor Laboratory Press, NY. 1989.

2 N.C. Tansil, F. Xie and Z.Q. Gao, *Anal. Chem.,* 2005, **77.** 126.

3 E. M. Genies and M. Larkowski, *J. Electroanal. Chem.,* 1987, **220,** 67.

4 B. Duke, E. M. Conwell and A. Paton, *Chem. Rev. Lett.,* 1986, **131,** 82.

5 Y. Ma, J. Zhang, G. Zhang and H. He, *J. Am. Chem. Soc.* 2004, **126,** 7097.

6 http://www.mirbase.org.