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Sensitive Detection of MicroRNA by Chronocoulometry and Rolling Circle Amplification on Gold Electrode

Bo Yao* [a], Yichen Liu [b], Miyuki Tabata[c], Huangtianzhi Zhu [a], Yuji Miyahara* [c]

[a] *Institute of Microanalytical Systems, Department of Chemistry, Zhejiang University, Hangzhou 310058, China. Fax: 86-571-88273572; Tel: 86-571-88273496; E-mail: yaobo08@zju.edu.cn*
[b] *Department of Chemistry, Lanzhou University, Lanzhou 730100, China.*
[c] *Department of Bioelectronics, Tokyo Medical and Dental University, Tokyo101-0062, Japan. Fax: 81-3-5280 8135; Tel: 81-3-5280 8095; E-mail: miyahara.bsr@tmd.ac.jp*

Materials and Methods

1. Primer and sequence

MicroRNA, DNA probes and primers were purchased from Sangong (Shanghai, China). The sequences of those DNA and miRNA oligoes were listed below in Table S1, among which mir-143 sequence was selected from the Sanger Center miRBase at http://microrna.sanger.ac.uk/sequences. All of those oligonucleotides were purified by HPLC and quantified with Nanodrop 2000 spectrophotometer from Thermo scientific.

Table S1 User designed oligomers and miRNA sequences

ID	Sequences
hsa-miR-143	5'-UGAGAUGAAGCACUGUAGCUC
hsa-miR-122	5'-UGGAGUGUGACAAUGGUGUUUG
hsa-miR-145	5'-GUCCAGUUUUCCCAGGAAUCCCU
hsa-miR-1	5'-UGGAAUGUAAAGAAGUAUGUAU
Circular probe	5'-phos-GTGGTTGTCTTCTCCTCAGCTCTATCGGATTTGTATCTCTCCT CAGCCTATCGGATTTGTATCTCTAAGCAGT
Probe1	5'-thiol-AAAAAAAAAAAAAAAAAAAAGAGCTACAG
Probe2	5'-TGCTTCATCTCAAAAAAAAAAAAAGACAACCACACTGCTTAGA

2. Fabrication of integrated gold electrode on polystyrene substrate

An integrated three-electrode biosensor was fabricated on polystyrene (PS) substrate by UV directed chemical plating technique. In brief, a piece of PS sheet (63×63×1.6 mm) was exposed to UV-lights (254 nm) emitted by a low-pressure mercury lamp for

about six hours. The exposed PS sheet was then immersed into 100 mM of phosphate buffer (pH 7.0) containing 360 mM of ethylenediamine (Sigma, China) and 50 mM of N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, Fluka, China) at room temperature for three hours. After rinsing with deionized water, the selectively aminated substrate was sequentially treated with 1 mM of HAuCl4 (Sinopharm, China) aqueous solution for 2.5 hours and 0.1 M of NaBH4 aqueous solution for 20 min. Thus, gold nano-particle catalysts were formed in the UV-exposure region on PS surface. Then the substrate was sonicated in 0.5 M KSCN solution for 20 min to remove non-specifically adsorbed gold species on the surface, so as to prevent non-specific over-plating in the following plating process. Finally, the activated PS was placed into a gold plating bath containing 0.125 M of Na_2SO_3 (Sinopharm, China), 0.6 M of methanal (Sinopharm, China) and 8 mM of $Na_3Au(SO_3)_2$ (Changzhou Institute of Chemical Research, Changzhou, China) for about three hours. After rinsing with deionized water, the PS gold electrode substrate was baked at 80^{o} C for 1 hour and ready for usage.

3. Modification of gold electrode with DNA probe.

Before use, gold electrodes were cleaned by electrochemical oxidation and reduction. One hundred cycles of cyclic voltammetry in 100 μL of 0.1 M H_2SO_4 solution were applying on the electrode using the following parametrers: potential range, -0.5 to 1.0V; scan rate, 1V/s. After being rinsed with Milli-Q water (Millipore, 18 mΩ•cm at 25ºC), the electrodes were dried with nitrogen. DNA modified surfaces were prepared by two-step immobilization procedure. In brief, the clean gold substrate was immersed in 1 μM solution of thiolated DNA probe in I-Buffer(10 mM Tris-HCl, 1 mM EDTA, 0.1 M NaCl, 10 mM TCEP, pH7.4) for 3 hours to overnight at room temperature. Rinsing with Milli-Q water, the electrodes were immersed in 1 mM of 6-Mercapto-1-hexanol (MCH, Aldrich, China) solution for at least 2 hours. Then the electrodes were immersed in 1 mg/mL of bovine serum albumin (BSA, Sangon, Shanghai, China) for 1 hour for covering the active groups on gold surface. Finally, the electrodes were rinsed with Milli-Q water and dried under nitrogen flow.

4. Preparation of circular probe

The circular probe used in our experiment for RCA amplification was circularized by CirLigase II ssDNA Ligase (Epicentre, Zhongbeilinge, Beijing, China) according to the manufacturer's procedure. Briefly, a mixure containing circular Probe (1 μM), reaction buffer, $MnCl_2$ (205 mM), and CirLigase II ssDNA Ligase (5U/uL) was incubated at 60° C for 1 h. After the ligation, the mixture was incubated at 80° C for 20 min to inactivate the ligase. The unligated linear sequence was digested by adding Exonuclease I (10U, New England Biolabs, China) and Exonuclease III (100U, New England Biolabs, China) and incubated at 37° C for 2 hours, followed by 80° C for 20 min to inactivate exonucleases. The ligated circular probe was purified using TIANquick oligo DNA purification Kit (Tiangen, Beijing, China) and quantified with Nanodrop 2000 spectrophotometer.

5. Total RNA isolation from human blood sample

Human blood sample was provided by Dr. Xuan Mu at Institute of Basic Medical Sciences Chinese Academy of Medical Sciences (School of Basic Medical Science Peking Union Medical College) and this study was approved by Chinese Academy of Medical Sciences Review Board on Human Rights Related to Human Experimentation (No.001-2014). The blood was reserved at -20°C in presence of anticoagulin. Before total RNA extraction, certain amount of synthesized mir-143 was spiked in the blood (from 10 fM to 10 nM). Total RNA was extracted using TIANprep Pure Blood Kit (Tiangen, Beijing, China) according to the manufacturer's protocol and the concentration of total RNA was determined by absorption readings at 260 nm on Nanodrop spectrophotometer.

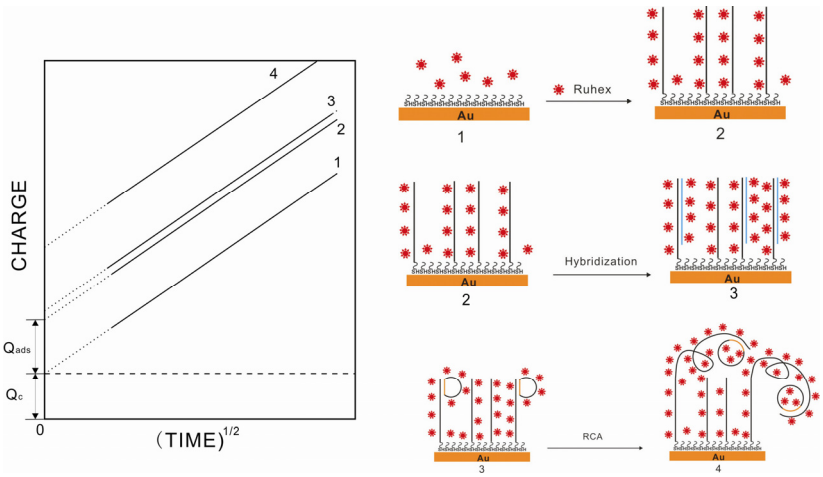
6. Hybridization and RCA amplification on gold electrode surface

The gold electrode modified with DNA probe1 was immersed in hybridization buffer containing 10 mM of tris, 50 μM of ruthenium(III) hexaammine (RuHex, Aldrich, China) and certain concentration of mir-143 and DNA probe2. Hybridization was performed at room temperature for one hour. The electrode was rinsed by milli-Q water and 10 mM of tris buffer for three times. Then RCA reaction mixture, containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 200 μg/mL BSA, 400 μM dNTP (Takara, Dalian, China) and 3 U of phi29 DNA polymerase (New England

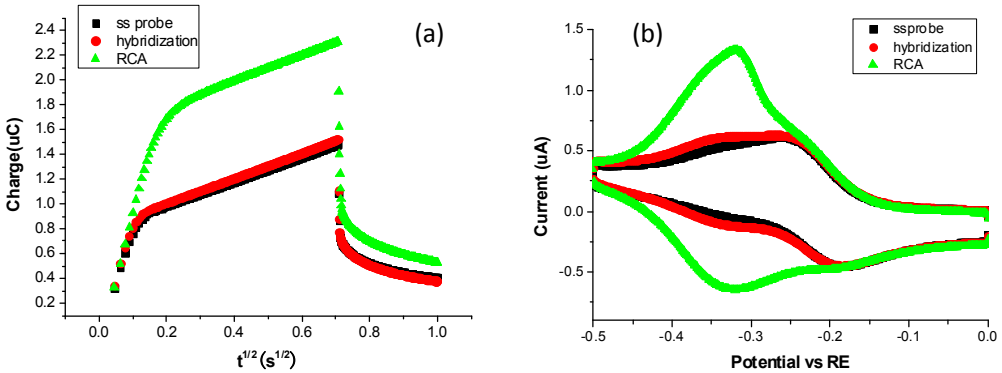
BioLabs, China) and 15 nM of circular probe, was transferred onto the electrode and incubated at 30 °C for a period of time (10 min-16 h).

7. **Chronocoulometry and cyclic voltammetry.**

Chronocoulometry (CC) and cyclic voltammetry (CV) was performed with AutoLab (PGSTAT302, Eco Chemie, Utrecht, Netherlands). The following parameters were employed. For CC measurement, two-step potential was employed from -500 mV to 0 mV with 500 ms duration. CV was also performed from -500 mV to 0 mV at 50 mV/s. The buffer for both CC and CV was 10 mM tris (pH 7.4) containing 50 µM of RuHex at room temperature.



FigureS1. Theoretical principle of measurement of immobilization, hybridization and RCA amplification through chronocoulometry.



FigureS2. Electrochemical behavior of DNA probe, target microRNA and RCA products modified gold electrodes. Representative chronocoulometry (a) and cyclic voltammetric (b) curves for gold electrodes modified with single-stranded DNA probe1

(black), hybridization with 1 nM of target microRNA and 10 nM of probe2 (red) and after RCA amplification (green) for two hours.

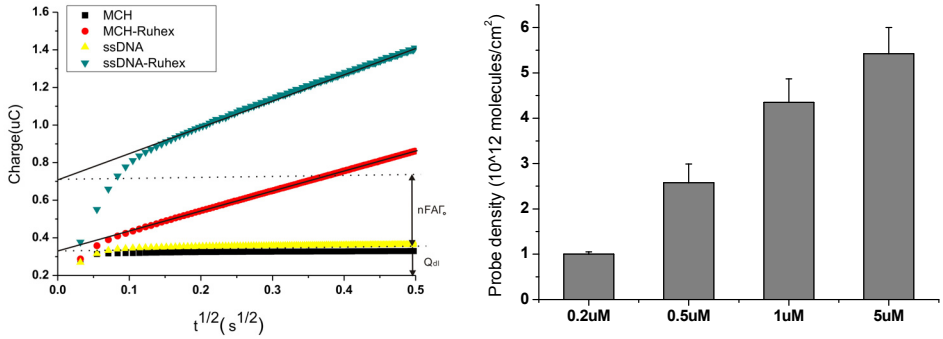


Figure S3 chronocoulometric response curves for thiolated ssDNA probe and MCH modified electrodes in the absence and presence of RuHex (50μM) (a); Surface density of SH-probe monolayer obtained by being incubated with different concentration of thiolated DNA probe1 (b).

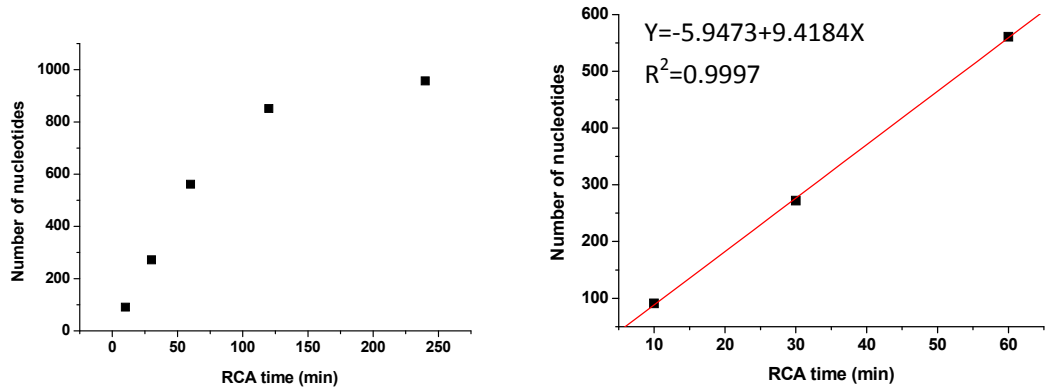


Figure S4. Average length of RCA products grew on the electrode surface depended on reaction time (10, 30, 60, 120 and 240 minutes). The average length was calculated based on equation (2) and (3) in the manuscript.

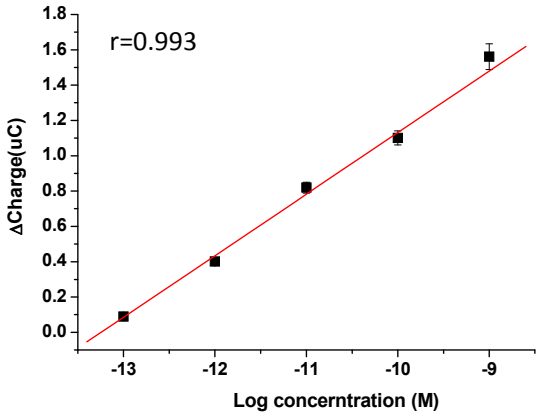


Figure S5. Standard curve of mir-143 detection based on RCA-CC assay. Stepwise diluted target microRNAs were put onto the DNA modified gold electrods. The concentration of mir-143 ranged from 100 fM to 1 nM. The concentration of DNA probe2 added for initiation of RCA amplification was 10 nM and RCA reaction was performed at 30 ºC for two hours.

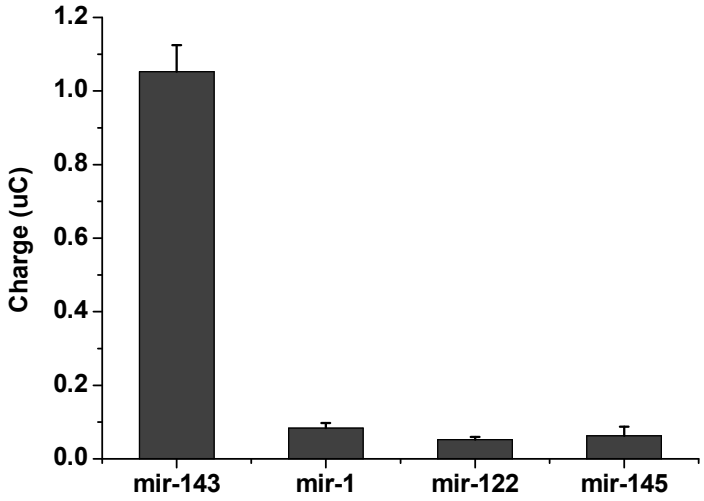


Figure S6. Selectivity of RCA-CC assay. 100pM of mir-143 and equal amount of other microRNAs including mir-1, mir-122 and mir-145 were added respectively and detected by RCA-CC assay. The concentration of DNA probe2 added for initiation of RCA amplification was 10 nM and RCA reaction was performed at 30 ºC for two hours.