An electrochemical signal "off-on" sensing platform for microRNA detection

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1. DNA and microRNA sequences used in this work

Table S1. DNA and microRNA sequences

	DNA and microRNA sequences（5'-3'）[a]
miRNA-21	UAG CUU AUC AGA CUG AUG UUG A
LNA integrated NAH probe (LNA-NAH)	SH-$(CH_2)_6$-GGC CGT CAA **C**AT CAG TC**T** GAT AAG C**T**A AAC ATG ATA CGG CC
Bridge DNA (S2)	CAA CTC TAG GGT CCC AGT GTA TCG CAA TGA CGA GAT CTG TAG TTC A
Bridge DNA (S1)	GGC CGA ATC ATG TTC GTC ATT GCG ATA CAC TGA GAT CTG TAG TTC A
Report DNA (S5)	GG**A** CCC T**A**G AGT **T**G-$(CH_2)_6$-SH
Report DNA (S4)	SH-$(CH_2)_6$-TG**A** ACT A**C**A GAT **C**T
Signal DNA (S3)	SH-$(CH_2)_6$-GCG GAA CAC TCA AG-biotin
Single-base mismatch miRNA	UAG CUU AUC ***G***GA CUG AUG UUG A
Three-base mismatch miRNA	U***U***G CUU AUC ***G***GA CUG AU***C*** UUG A
Non-complementary miRNA	GUA AGG CAU CUG ACC GAA GGC G

a: Nucleotide mismatches were indicated as italic and bold letters, locked nucleic acid bases were indicated as underlined and bold letters.

2. Preparation of AuNPs modified with reported DNA and signal DNA

AuNPs modified with reported DNA and signal DNA were prepared according to previous reports with some minor revision.[1, 2] In brief, 3 μL 7.1×10^{-6} M reported DNA (S4 or S5), 10 μL 1.0×10^{-5} M signal DNA (S3), 1 μL 10 mM acetate buffer (pH 5.2) and 1 μL 10 mM 3.3×10^{-6} M TCEP were added into EP tube and incubated for 1 h without light. After that, 1 mL freshly prepared gold nanoparticles was added and shook gently for 16 h without light. Then, the obtained DNA-AuNPs conjugates were aged in 10 mM NaCl and 10 mM acetate buffer for 24 h. Excess reagents were removed by centrifugation at 12000 rpm for 30 min. The red precipitate was washed, and centrifuged repeatedly for three times. The resulting nanoparticles were dispersed into 1 mL of 0.1 M PBS (pH 7.4) containing 10 mM NaCl, and it was stored at 4 °C.

3. Preparation of bridge DNA

The bridge DNA was prepared according to previous reports with minor modification.[1, 3] 5 μL of 1.0×10^{-5} M S1 solution and 5 μL of 1.0×10^{-5} M S2 solution were added into 1 mL 0.1 M PBS (pH 7.0). And the mixture was stirred for 4 h at 37.5 °C to complete the hybridization reaction. The prepared bridge DNA was kept in PBS at 4 °C until use.

4. Preparation of bridge DNA integrated AuNPs-bio-bar codes signal amplification unit

The bridge DNA integrated AuNPs-bio-bar codes signal amplification unit was prepared as follows.[1] Briefly, 300 μL S4 and S3 labeled AuNPs, 150 μL S5 and S3 labeled AuNPs, and 300 μL bridge DNA were mixed. After shaking gently for 24 h

without light, the solution was allowed to stand for another 48 h, followed by centrifugation for 30 min at 10000 rpm to remove excess reagents. Following removal of the supernatant, the red precipitate was washed with 1 mL of 10 mM PBS (pH 7.4) containing 10 mM NaCl, recentrifuged, and then redispersed in 1 mL of 10 mM pH 7.4 PBS containing 10 mM NaCl. The prepared signal amplification unit was stored at 4 °C.

5. Preparation of miRNA biosensor based on nanomaterials, LNA-NAH probe and bridge DNA integrated AuNPs-bio-bar codes signal amplification unit

The planar gold electrode was polished with 0.03 μm alumina powder on micro-cloth pad and rinsed thoroughly with redistilled deionized water. Then the polished gold electrode was washed successively with redistilled deionized water, anhydrous ethanol and redistilled deionized water in an ultrasonic bath. After dried with nitrogen blowing, the planar gold electrode was dipped into piranha solution (H_2SO_4/H_2O_2, 7:3, v/v) for 10 min and washed successively with redistilled deionized water, anhydrous ethanol and redistilled deionized water in an ultrasonic bath. Prior to modification, the planar gold electrode was scanned by cyclic voltammetry from -0.2 to 1.6 V in 0.5 M H_2SO_4 until a voltammogram characteristic of the clean polycrystalline gold electrode was established. Then, 5 μL of graphene dispersion (2 mg/mL) was dripped on the electrode surface and dried under infrared lamp. After washed with redistilled deionized water, the electrode was immersed into 3 mM $HAuCl_4$ solution containing 0.1 M KNO_3. Subsequently, the AuNPs were electrochemically deposited on the electrode surface at -0.2 V for 600 s under

single-potential mode. Finally, the electrode was washed with redistilled deionized water and dried with room temperature.

Subsequently, the electrode was immersed into an immobilization buffer solution containing 1.0×10^{-8} M probe and 5.0×10^{-8} M MPA for 22 h to obtain a well-aligned probe monolayer, followed by rinsed three times with 10 mM Tris-HCl to remove the unspecific adsorbed probe and MPA. Then the electrode was immersed into miRNA hybridization buffer containing a certain concentration of miRNA-21 for 1.5 h at room temperature in a humidified chamber to complete the hybridization event. After that, the electrode was rinsed three times with 0.1× SSC for 15 min to remove the un-hybridized target miRNA-21. Subsequently, the modified electrode was immersed into DNA hybridization buffer containing bridge DNA integrated AuNPs-bio-bar codes signal amplification unit for 2.5 h and rinsed three times with 0.1× SSC for 15 min to remove nonspecifically adsorbed sequences.

Finally, the modified electrode was incubated in 0.05% PEG-3350 solution (prepared in 0.1 M PBS) for 1 h to prevent any possible nonspecific binding. After the electrode was rinsed three times with 0.1 M PBS (pH 7.0) for 15 min, 5 μL streptavidin-HRP (1:100) was dripped on the electrode surface and incubated for 30 min at room temperature in humid conditions. The electrode was then rinsed three times with 0.01 M PBS and dried with room temperature. The obtained electrode was noted as HRP/AuNPs-Barcode/miRNA-NAH/AuNPs/Graphene/Au. For comparison, HRP/AuNPs-Barcode/miRNA-NAH/Au and HRP/AuNPs-Barcode/miRNA-NAH/AuNPs/Au were also prepared with the same

process.

6. Characterization of AuNPs/Graphene/Au

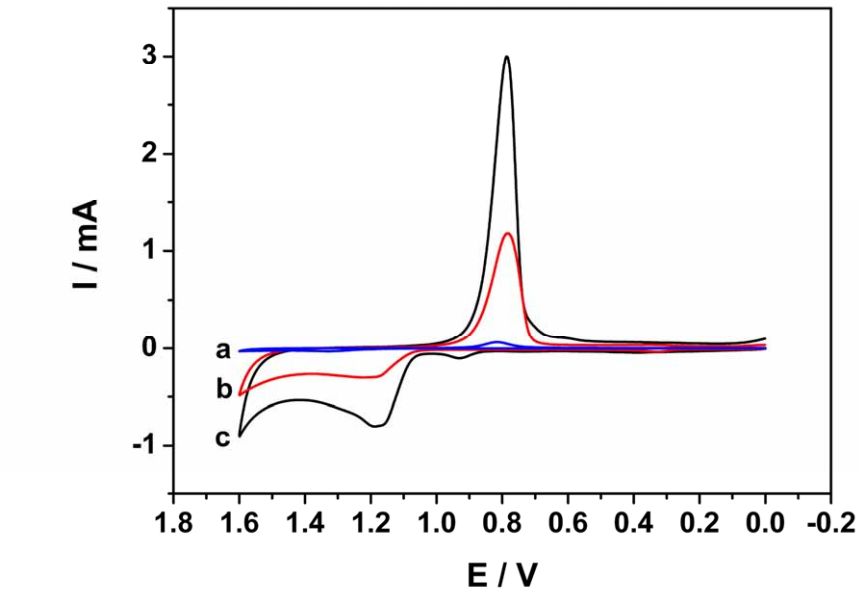

Fig. S1 Cyclic voltammograms of planar Au electrode (a), AuNPs/Au (b) and AuNPs/Graphene/Au (c) in 0.5 M H_2SO_4. Scan rate, 100 mV/s.

For miRNA detection using the strategy proposed in this work, one of the crucial factors was LNA integrated NAH probe immobilization amount, which mainly depended on the increased surface of AuNPs/Graphene/Au. Therefore, it was characterized by CV in 0.5 M H_2SO_4. For control, the planar Au electrode and AuNPs/Au with the same geometric surface area were also characterized under the same conditions. As seen in Fig. S1, the CV of AuNPs/Graphene/Au (curve c) showed a significant larger reduction peak at about 0.786 V for gold oxide compared with the planar Au electrode (curve a) and AuNPs/Au (curve b), indicating that AuNPs/Graphene/Au has a much larger active surface. Assuming a specific charge of 386 $\mu C/cm^2$ for the gold oxide reduction,[4] the active surface area of

AuNPs/Graphene/Au was calculated to be 3.997 cm^2, while the corresponding planar Au electrode and AuNPs/Au were 0.1547 and 0.7148 cm^2, respectively. The 25.84-fold and 5.59-fold enhancement to the planar Au electrode and AuNPs/Au were obtained, which can be attributed to the large specific area and irregular structure of graphene. The active surface area of AuNPs/Graphene/Au was also higher than some modified Au electrodes reported previously, such as nanoporous gold electrode (1.097 cm^2),[5] macroporous ultramicroelectrodes (3.3 cm^2),[4] dendritic gold nanostructure modified gold electrode (8.3-fold to planar gold electrode) [6] and AuNPs/multilayer-polyelectrolyte/Au (3.3-fold to planar gold electrode).[7] According to the equation of $f = n\pi(4/3)^{1/2}$,[4] where f is the enhancement factor and n is the number of pore layers, the number of pore layers could be calculated as 7.127.

7. Optimization of experimental conditions

Because the high hybridization efficiency can improve the determination sensitivity, the effect of probe concentration, probe immobilization time, miRNA hybridization time and reported DNA hybridization time on the reduction peak current of benzoquinone was investigated by chronoamperometry.

Figure S2A showed the influence of probe concentration on miRNA-21 hybridization event. The reduction signal of benzoquinone enhanced with probe concentration increasing from 10^{-11} to 10^{-8} M. The maximum current was obtained at 10^{-8} M. With increasing the NAH probe concentration, more probes can be immobilized on the electrode. However, an excess of probes on the electrode surface can improve the difficulty in unfolding the loop-stem structure of probe due to the

steric hindrance effect, which can further decrease the hybridization efficiency. Therefore, 10^{-8} M probe concentration was selected. Figure S2B showed the effect of probe immobilization time on the reduction current of benzoquinone. The current increased gradually with extending the immobilization time from 12 to 22 h. Then the current decreased reversely. Considering the determination sensitivity, 22 h was selected as the optimal condition. The effect of miRNA-21 hybridization time was investigated by varying the hybridization time from 30 to 180 min (Figure S2C). With the time increasing from 30 to 90 min, the reduction current increased gradually. Then, the current increased very slightly with further extending the hybridization time. To ensure the relative high hybridization and determination efficiency, 90 min was selected throughout our experiments. Figure S2D showed the effect of hybridization time of the reported DNA loaded on bridge DNA-AuNPs-Bio bar code signal amplification unit with NAH probe on the reduction current of benzoquinone. Similar to target miRNA-21, the current increased gradually from 30 to 150 min, and then the increase tendency slowed down. Therefore, 150 min was selected as the optimum hybridization time.

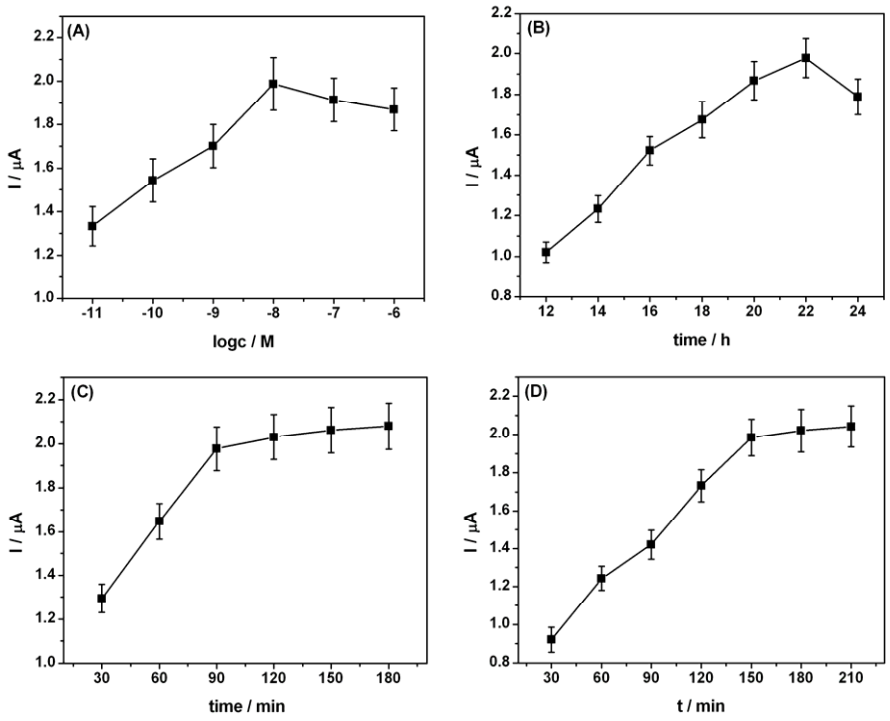

Figure S2. Effect of probe concentration (A), probe immobilization time (B), miRNA-21 hybridization time (C) and reported DNA hybridization time (D) on the reduction peak current of benzoquinone.

8. Regeneration and stability of the fabricated DNA biosensor

Regeneration is useful for continuous monitoring of the target miRNA-21 in future research. We tested the possibility to reuse the biosensor by regenerating the original state of the hairpin probes. It was found that the fabricated biosensor could be regenerated 3 times with about 19.34% loss of the original signal by dipping the electrode in hot water (80 °C) for 10 min, followed by a rapid cooling in an ice bath for 10 min. The signal attenuation seemed to be attributed to the loss of thiolated probes on the electrode surface. The biosensor's lifetime was also especially important for evaluating the performance of biosensors. The activity of the biosensor could retain its 91.38% original value after stored for 2 weeks in refrigerator.

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