Electronic Supplementary Information

Bipedal DNA Walker Mediated Enzyme-free Exponential Isothermal Signal Amplification for Rapid Detection of MicroRNA

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Reagents and materials

HPLC-purified DNA and RNA oligonucleotides were synthesized by Sangon Biotech Co., Ltd (Shanghai, China). Streptavidin (SA) was purchased from Solarbio Science & Technology Co., Ltd (Beijing, China). Bis(2,2-bipyridyl)(4-methyl-4-carboxypropyl-2,2-bipyridyl) ruthenium(II) hydrochloride ($Ru(mcbpy)(bpy)_2Cl_2$) was purchased from SunaTech Inc. (Soochow, China). Branched polyethyleneimine (PEI, Mw 70k Da) was purchased from Macklin Inc. (Shanghai, China). Polyacrylic acid (PAA, 200k Da), polyvinylpyrrolidone (PVP), tris(2-carboxyethyl)phosphine (TCEP), 6-Mercapto-1-hexanol (MCH), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and N-Hydroxysuccinimide (NHS) were purchased from J&K Scientific Ltd. (Beijing, China). Ammonium fluorotitanate was purchased from Titan Scientific Co., Ltd (Shanghai, China). Other analytical grade (A.R.) reagents were purchased from Kelong chemical engineering company (Chengdu, China). TE buffer (10 mM Tris-HCl, 1.0 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0) was used for the storage of all the oligonucleotides. STE buffer (10 mM Tris-HCl, 150 mM NaCl, 1.0 mM EDTA, pH 8.0) was used as the reaction buffer for the reactions of oligonucleotides. 0.1 M phosphate buffered saline (PBS, pH 7.4) containing 0.1 M KCl was prepared for electrolyte solution. The experiments were carried out under RNase-free environment. The sequences of the oligonucleotides used in this work were listed in Table S1.

Table S1 Sequences of the oligonucleotides.

Name	Sequence (5' to 3')
miR-21	UAG CUU AUC AGA CUG AUG UUG A
H1	TCAACATCAGTCTGATAAGCTACCATGTGTAGATAGCTTATCAGA CTTTTTTTTTTTCAGTAGTTCGTGATCGAC
H2	TAAGCTATCTACACATGGTAGCTTATCAGACTCCATGTGTAGATT TTTTTTTTCAGTAGTTCGTGATCGAC
CP	HS-$(CH_2)_6$-TTTT CAG TAG TCA ACA TCA GT CGA TCA CGA ACT ACT G
ST	TAG CTT ATC AGA CTG ATG TTG A
AP	TTCG TGA TCG CTG ATA AGC TA
H3	Biotin-TTTTCAG TAG TTC GTG ATC GAC TGA TGT TGA CT ACT G

Apparatus

The ECL measurements were performed on a model MPI-EII ECL analyzer (Xi'An Remax Electronic Science & Technology Co. Ltd., Xi′An, China). Electrochemical measurement was carried out with a CHI760C electrochemistry workstation (Shanghai Chenhua Instruments, China). Three-electrode system composed of a Ag/AgCl electrode (saturated KCl) (the reference electrode), a platinum wire (auxiliary electrode) and a modified glassy carbon electrode (GCE, Φ = 3 mm) (the working electrode) was used for ECL and electrochemistry measurements in the experiment. The surface appearance of nanomaterials was observed by Hitachi S4800 scanning electron microscopy (Hitachi, Japan) at an acceleration voltage of 25.0 kV. The crystalline structure was characterized by Bruker D8 Advance X-ray diffractometer (Bruker, USA). Elemental analysis was carried out on an ESCALAB 250Xi X-ray photoelectron spectrometer (Thermo Scientific, USA). Native polyacrylamide gel electrophoresis was performed with Mini-PROTEAN® Tetra electrophoresis Chambers and Gel Doc™ XR+ System (Bio-Rad, USA).

Synthesis of the TiO_2@PEI-Ru@SA ECL signal probes

The preparation of TiO_2 nanospheres were according to the reported method.[1] Briefly, 40 mL aqueous solution containing 0.05 g $(NH_4)_2TiF_6$, 0.5 g polyvinyl pyrrolidone (PVP), and 0.5 g urea was heated in an autoclave at 180 °C for 8 h. After cooling down to room temperature, white precipitate was obtained and washed with deionized water and absolute ethanol, successively, for 5 times. Finally, white TiO_2 powders were collected after drying at 60 °C for 2 h.

PEI-Ru complex was synthesized *via* a brief crosslinking process. First, 1.0 mg Bis(2,2-bipyridyl)(4-methyl-4-carboxypropyl-2,2-bipyridyl) ruthenium(II) hydrochloride ($Ru(mcbpy)(bpy)_2Cl_2$) was dissolved in 1.0 mL MES buffer (pH 6.0) containing 50 mg/mL 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and 50 mg/mL N-Hydroxysuccinimide (NHS) followed by vertical mixing for 30 min at room temperature. Then, 8.0 mL polyethyleneimine (PEI) solution (1% w/v) was added into the solution and vertically mixed for 2 h at room temperature to obtain the Ru-PEI complex. The resultant solution was directly used to prepare TiO_2@Ru-PEI nanospheres without purification.

TiO_2 nanospheres were encapsulated with Ru-PEI *via* a typical layer-by layer assembly process.[2] Briefly, 1 mg TiO_2 was dispersed in 1 mL deionized water and then supplied into the Ru-PEI complex solution. Under continuous stirring, 1 mL phosphate buffered saline (PBS, 0.1 M, pH 7.4) was dropwise added into the solution. After stirring for 30 min, the solution was centrifuged under 12000 r/min (5 min). Orange precipitate (TiO_2@PEI-Ru) was dispersed in PBS and then dropwise supplied into (1

mg/mL) PAA solution under ultraphonic dispersion. After centrifugation and washing, the precipitate was dispersed in 1 mL PBS again. The above process was repeated for 3 times, followed by washing with deionized water and ethanol successively to remove the redundant reagents.

To activate the carboxyl groups, TiO_2@PEI-Ru nanocomposite was dispersed in 1 mL MES buffer containing 50 mM EDC and 50 mM NHS. After vertically mixed at room temperature for 30 min, the precipitate was collected by centrifugation and washed with MES buffer for 3 times. Then, the obtained nanoparticles was dispersed in 900 μL deionized water, followed by adding 100 μL streptavidin solution (SA, 1 mg/mL). After vertically mixed for 2 h, SA molecules were well labeled on TiO_2@PEI-Ru nanospheres (TiO_2@PEI-Ru-SA).

Fabrication of the biosensor

Single stranded DNAs CP (500 nM), AP (600 nM) and ST (600 nM) were dissolved in STE buffer and annealed from 95 °C to 25 °C in 210 min to prepare the Y junction structure (CP-AP-ST). With the addition of 1 mM tris(2-carboxyethyl)phosphine (TCEP), the CP-AP-ST solution was stored at 4 °C for next step. The cleaned mirror-like electrode surface of glassy carbon electrode (GCE, $\Phi = 3$ mm) was obtained according to the literature.[3] Subsequently, the electrode surface was electrodeposited with Pt nanoparticles (Pt NPs) to obtain Pt NPs modified GCE (Pt NPs/GCE) by immersed in 1% H_2PtCl_6 solution with constant potential at -0.25 V for 30 s. After rinsing with deionized water and drying in nitrogen atmosphere, the Pt NPs/GCE was incubated with 10 μL the prepared CP-AP-ST solution for 12 h at room

temperature to fabricate CP-AP-ST on the electrode (Track/Pt NPs/GCE). Finally, the biosensor finished fabrication by block the nonspecific binding sites with MCH (1 mM) to obtain the sensing interface MCH/Track/Pt NPs/GCE.

ECL measurement of the biosensor

For the detection of miR-21, the sample solution (5 μL) and a mixed solution (5 μL) containing H1 (200 nM), H2 (200 nM) and biotin-HP (200 nM) was together dropped on the sensing interface of the biosensor. After incubating for 40 min, 0.1 M PBS (pH 7.4) was used to rinsing the biosensor to remove unbound reagents. Then, TiO_2@PEI-Ru@SA (10 μL) was incubated with the biosensor for 10 min, followed by rinsing with PBS again. Finally, the biosensor was applied with the scanning potential from 0.2 to 1.2 V at scan rate of 200 mV/s in 0.1 M PBS (pH 7.4) for ECL measurement (photomultiplier high-voltage 800 V, magnitude 3). To plot the calibration curve, standard sample of miR-21, instead of the sample solution, was tested by the biosensor with different concentrations.

ECL co-reaction acceleration of TiO_2 nanopheres to PEI-Ru complex

To confirm the ECL co-reaction acceleration of TiO_2 nanopheres to PEI-Ru complex, the ECL intensities of the PEI-Ru complex were measured with bare GCE and TiO_2 nanopheres modified GCE, respectively. As shown in Fig. S1, after modifying TiO_2 nanopheres on the electrode surface, the ECL intensity notably increased from 1410 a.u.to 4189 a.u.. The result indicated that the TiO_2 nanopheres could effectively improve the ECL of PEI-Ru complex.

According to our previous work[4], the mechanism could be explained by following processes. With the effect of the applied voltage, the electrons tunnelled from the valence band of TiO_2 to the conduction band.[5] Meanwhile, a hole was generated in the valence band, which gained electrons from the species on the surface of TiO_2.[6] In this work, the hole of TiO_2 obtained electrons from PEI-Ru to accelerate the generation of $PEI^{+\bullet}$-Ru(III), resulting in more $PEI^{\bullet}$-Ru(III) in unit time through the deprotonation process. Hence, a significant enhancement of reaction rate between the $PEI^{\bullet}$-Ru(III) was obtained, and as a result the ECL emission increased (Scheme S1).

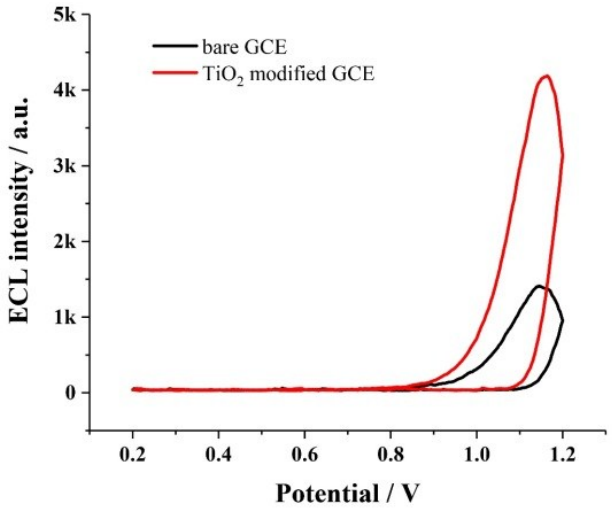
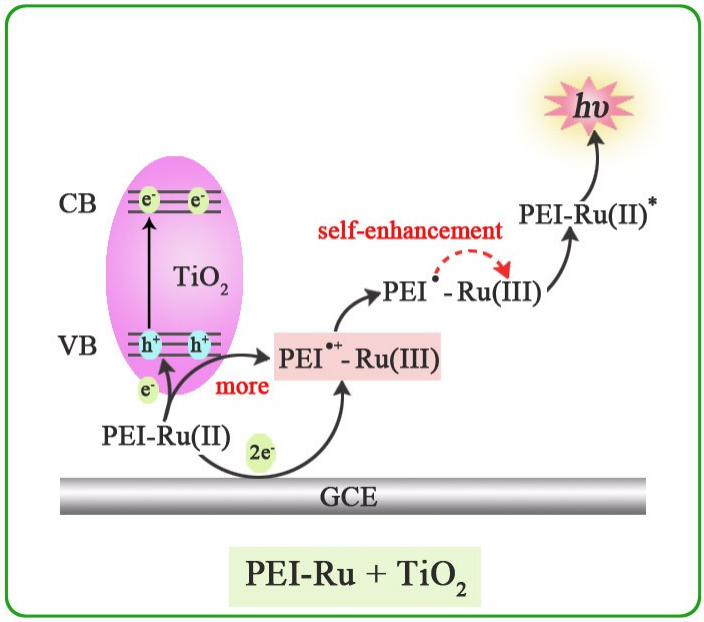


Fig. S1 ECL intensities of PEI-Ru complex with different working electrodes.



Scheme S1 Possible ECL mechanisms of the PEI-Ru complex + TiO_2 system.

SEM characterization of electrodeposited Pt NPs

The morphology and size of the electrodeposited Pt nanoparticles (Pt NPs) have been characterized by scanning electron microscope (SEM). The Pt NPs were electrodeposited on the well-polished interface of a dismountable glassy carbon electrode. As displayed in Fig. S2, the typical SEM image demonstrated that Pt NPs exhibited a well-defined shape of flower-like structures with a diameter of 250 nm, indicating the successfully electrodeposition of Pt NPs.

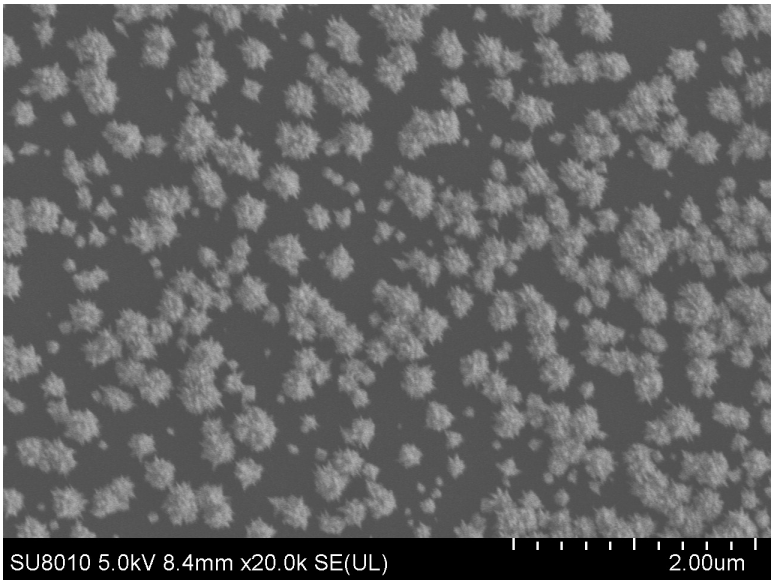


Fig. S2 SEM image of electrodeposited Pt NPs.

Electrochemical impedance spectroscopy characterization of biosensor fabrication

To confirm the fabrication of the ECL biosensor, electrochemical impedance spectroscopy (EIS) was measured with different electrode in 5.0 mM $[Fe(CN)_6]^{3-/4-}$ solution. As shown in Fig. S3, bare GCE presented a small semicircle (curve a), while it was almost in a straight line after the modification of Pt NPs (curve b). When tracks (CP-AP-ST) were assembled on the Pt NPs modified electrode (curve c), the resistance prominently increased because the Y junction structure of the tracks significantly impeded the electron transfer. After MCH blocking the nonspecific binding sites, the resistance further increased (curve d). Then the mixture of H1, H2, biotin-HP and miR-21 was dropped onto the modified electrode to conduct autonomous DNA walking, which presented a decreased resistance (curve e). Finally, when the modified electrode was incubated with TiO_2@PEI-Ru@SA composite, the obvious resistance increase was observed (curve f). These EIS results verified the successful fabrication of the ECL biosensor.

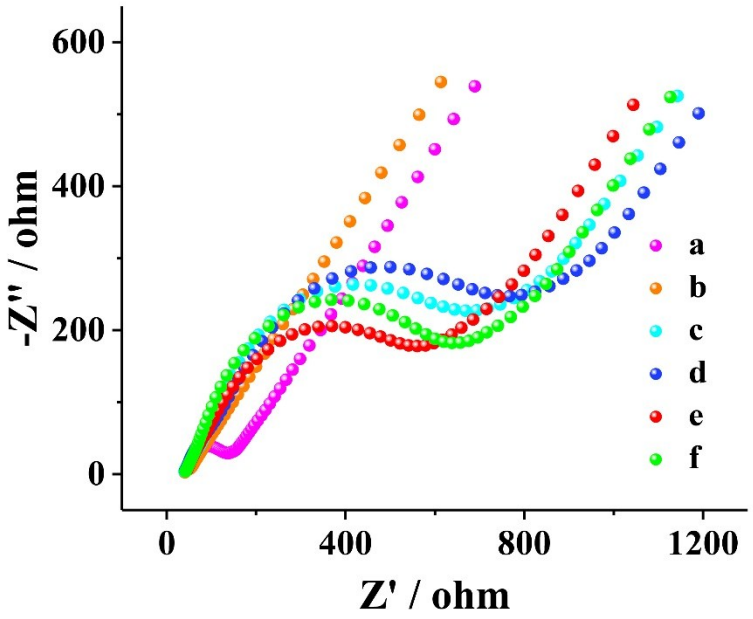
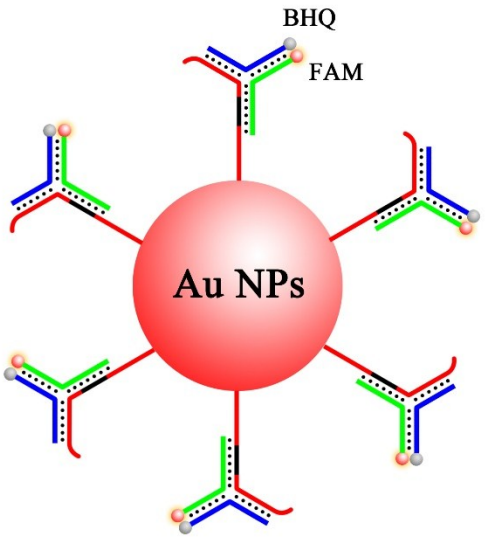


Fig. S3 EIS at the bare GCE (a), Pt NPs/GCE (b), Tracks/Pt NPs/GCE (c), MCH/Tracks/Pt NPs/GCE (d), DNA walking/MCH/Tracks/Pt NPs/GCE (e), TiO_2@PEI-Ru@SA/DNA walking/MCH/Tracks/Pt NPs/GCE (f). The EIS experiments was implemented in 5.0 mM $[Fe(CN)_6]^{3-/4-}$ solution.

Kinetics study for the proposed exponential amplification

In order to investigate the reaction kinetics of the proposed bipedal DNA walker mediated enzyme-free exponential isothermal signal amplification, a nanomachine was constructed by fabricating the tracks on gold nanoparticles (Au NPs, 13 nm) to monitor the DNA reactions by recording the fluorescence. As shown in Scheme S2, ST and AP were labeled with FAM and BHQ, respectively. Consequently, the fluorescence was quenched unless DNA walking was initiated. Fig. S4A displayed the real time fluorescence curves of the nanomachine in response to miR-21 at different concentrations. Curve a presented the real time fluorescence of the nanomachine in PBS, which exhibited stably weak fluorescence, indicating the good stability of the tracks. Then, H1 (10 nM), H2 (10 nM), and biotin-HP (10 nM) was added into the solution, while the fluorescence intensity still kept a low level (curve b). Significantly, after 10 pM miR-21 was supplied into the above solution, the fluorescence presented notable increase over a period of 1h (curve c), manifesting the sensitive response of the nanomachine to miR-21. Furthermore, when the concentration of miR-21 increased to 100 pM, the fluorescence presented much faster increase (curve d). To verify the exponential signal amplification, the reaction rate (first derivative of curve d) was calculated (Fig. S4B). Significantly, the reaction rate increased at the early stage and then decrease, which showed a typical characteristic of exponential amplification.



Scheme S2 Schematic diagram for the tracks fabricated on Au NPs.

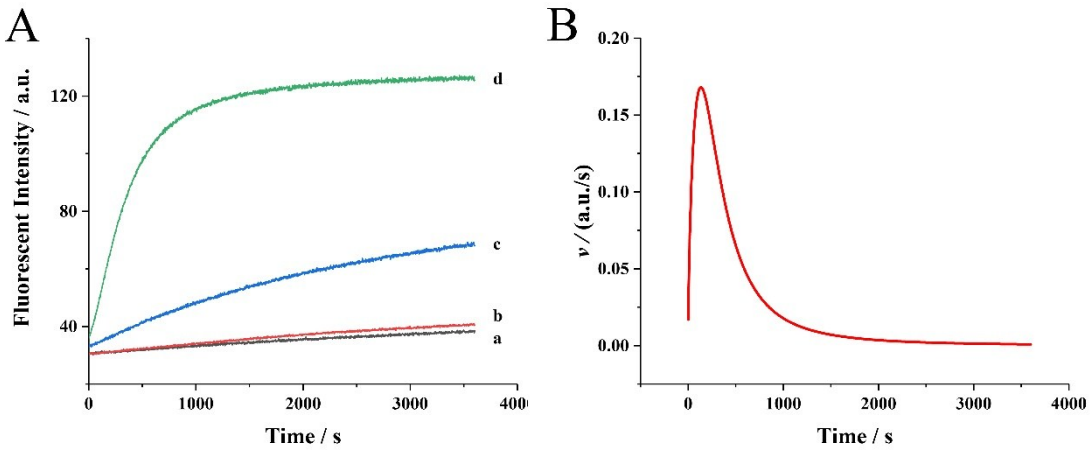


Fig. S4 (A) Real time fluorescence in response to various concentrations of miR-21. (B) Reaction rate curve of the bipedal DNA Walker mediated enzyme-free exponential isothermal signal amplification.

Rationality analysis of the bipedal DNA walker mediated exponential signal amplification strategy

To confirm the necessity of the components in the bipedal DNA walker mediated exponential signal amplification strategy, a series of control experiments ware performed by controlling the absence of different components (Fig. S5), including miR-21, H1, H2, and biotin-HP. Curve a showed the ECL response of the modified electrode (MCH/Tracks/Pt NPs/GCE) directly incubating with TiO_2@PEI-Ru@SA probes (absence: miR-21, H1, H2, and biotin-HP), exhibiting an ECL signal about 648 a.u. ascribed to the nonspecific absorption of the TiO_2@PEI-Ru@SA probes. Curve b showed the ECL response of the modified electrode successively incubating with the mixture of H1, H2 and biotin-HP (absence miR-21) and TiO_2@PEI-Ru@SA probes, exhibiting an ECL signal about 773 a.u.. Curve c showed the ECL response of the modified electrode successively incubating with the mixture of miR-21 and biotin-HP (absence: H1 and H2) and TiO_2@PEI-Ru@SA probes, exhibiting an ECL signal about 744 a.u.. Curve d showed the ECL response of the modified electrode successively incubating with the mixture of miR-21, H1 and H2 (absence biotin-HP) and TiO_2@PEI-Ru@SA probes, exhibiting an ECL signal about 694 a.u.. All these experiments presented very low ECL signals. However, when the modified electrode was successively incubated with the mixture of H1, H2, miR-21 and biotin-HP (no absent component) and TiO_2@PEI-Ru@SA probes, a strong ECL signal of 11115 a.u. was observed (curve e). The results indicated that all the components (including miR-21,

H1, H2, and biotin-HP) were essential to the normal working of the bipedal DNA walker mediated exponential signal amplification.

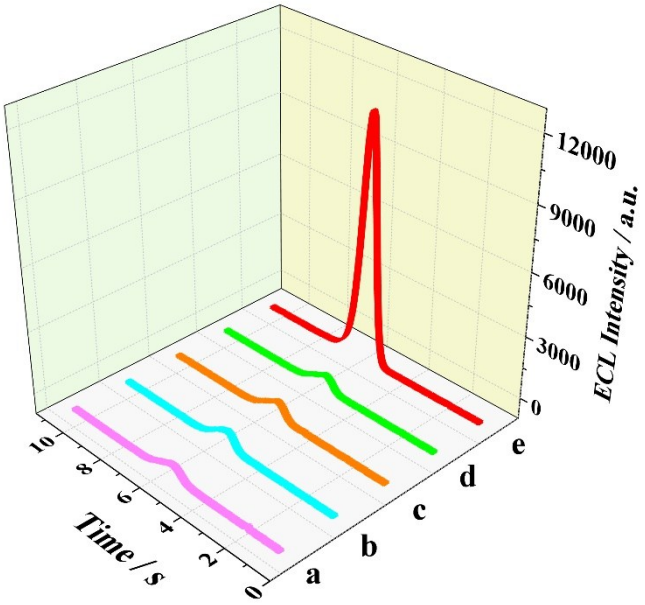


Fig. S5 ECL responses of the modified electrodes (MCH/Tracks/Pt NPs/GCE) incubated with different components: (a) none, (b) H1, H2 and biotin-HP, (c) miR-21 and biotin-HP, (d) H1, H2 and miR-21, (e) H1, H2, miR-21 and biotin-HP, and the further incubated with TiO_2@PEI-Ru@SA probes. The concentration of target miR-21 was 100 fM.

References

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