Supporting Information

Ratiometric electrochemiluminescent cytosensor based on polyaniline hydrogel electrodes in spatially separated electrochemiluminescent systems

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Section 1: Experimental Section

Materials and Reagents

Luminol, aniline, phytic acid, tripropylamine (TPA), 3-aminopropyl)triethoxysilane (APTES), potassium peroxodisulfate ($K_2S_2O_8$), hyaluronic acid (HA), tris(2,2 ′-bipyridyl) ruthenium(II) chloride hexahydrate ($Ru(bpy)_3Cl_2·6H_2O$), folic acid (FA) ethyl(dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were bought from Sigma-Aldrich (USA). Hydrogen tetrachloroaurate trihydrate ($HAuCl_4·3H_2O$) was achieved from Shanghai Yuanye Biotechnology Co., Ltd. (China). Melamine, ammonium persulfate ($(NH_4)_2S_2O_8$), triton X-100, tetraethyl orthosilicate (TEOS), ammonia solution ($NH_3·H_2O$, 25%), and 1-hexanol were obtained from Aladdin Industrial Co., Ltd. (Shanghai, China). Epidermal growth factor (EGF) was purchased from Cell Signaling Technology (USA). All solutions were prepared with deionized water (18.2 MΩcm).

Apparatus

The ECL detection was carried out on a MPI-E detection system (Xi'an Rimax, China) with three glassy carbon electrodes (GCE, diameter of 3 mm) as working electrodes, three Ag/AgCl (saturated KCl) electrodes as reference electrodes and auxiliary electrodes. Scanning electron microscopy (SEM) tests were operated on a Hitachi S-4800scanning electron microscope (Hitachi Co., Japan).Transmission electronmicroscopy (TEM) images were taken using a JEOL-2100 instrument (JEOL, Japan). UV-vis absorption was performed on a NANODROP 2000c spectrophotometer (Thermo Scientific, USA).

Preparation of polyaniline hydrogel (PAniH) modified electrode

The method for the preparation of polyaniline hydrogel has been reported [1]. Solution A: 2 mL H_2O and 0.921 mL phytic acid (50% w/w in water) were mixed up. Then adding 0.458 mL aniline (5 mmol), we sonicated the mixture 1 min to make it homogeneous. Solution B: 0.286 g ammonium persulfate (1.25 mmol) was dissolved in 1 mL H_2O. The solution A and B were put in refrigerator and cooled to 4 °C. 5 μL mixed solution of solution A and B was coated on the surface of a pretreated GCE quickly. The mixture

polymerized and cross-linked to form PAniH in 3 min. After placing in refrigerator at 4 °C for 30 min, the electrode was immersed in ultrapure water for 30 min to remove excess ions and any organics.

Synthesis of luminol-functioned gold nanoparticles (Lu-Au NPs)

All glassware used in the following procedures was cleaned in a bath of freshly prepared 3:1 (v/v) HNO_3-HCl, rinsed thoroughly in redistilled water, and dried prior to use. The Lu-Au NPs were prepared via the reduction of $HAuCl_4$ by luminol according to the previous work [2,3]. Luminol serves as both reducing and stabilizing agents for AuNPs. The size of luminol-reduced AuNPs are in a range of 14 ~ 35 nm, which can be adjusted by changing the amount of luminol. However, they contribute to the ECL without big changes. In this work, we used the AuNPs with a size of 20 nm. In detail, a 100 mL portion of $HAuCl_4$ (0.01% w/w) solution was heated to boiling. While stirring vigorously, 1.80 mL of 0.01 M luminol stock solution was added rapidly. The solution was maintained at the boiling point for 30 min, during which time a color change from yellow to black to purple was observed before a wine-red color was reached. The heating source was removed, and the colloid was kept at room temperature for another 20 min and then stored at 4 °C.

Synthesis of g-C_3N_4 nanosheet

The bulk g-C_3N_4 material was prepared according to the previous report [4,5]. Melamine was heated at 550 °C in a muffle furnace for 4 h with a ramp of 2.3 °C·min^{-1}. The product was cooled to room temperature and then grounded to powder. 1.0 g of g-C_3N_4 powder and 100 mL of 4 M HNO_3 were refluxed at 120 °C for 24 h. The refluxed product was centrifuged at 12,000 rpm and then washed with deionized water until pH reached 7.0. Finally, the product was dried in a vacuum oven for 12 h at 35°C to obtain the carboxylated g-C_3N_4. 200 mg of carboxylated g-C_3N_4 was dispersed into 100 mL distilled water by sonicating for 12 h. Afterward, the obtained suspension was centrifuged at 8000 rpm for 15 min to remove the unexfoliated bulk g-C_3N_4 powder. Subsequently, the supernatant was concentrated by heating and then diluted to 5.0 mL with distilled water for future use.

Synthesis of $Ru(bpy)_3^{2+}$-doped silica nanoparticles ($RuSiO_2$ NPs)

$RuSiO_2$ nanoparticles were synthesized as previously described with a little modification [6]. First, 1.77 mL of Triton X-100 were mixed with 7.5 mL of cyclohexane and 1.8 mL of n-hexanol at 25 °C. After 30 min of stirring, 340 μL of 0.04 M $Ru(bpy)_3^{2+}$ aqueous solution was added into the mixture. After 30 min of stirring, 100 μL of TEOS was added into the mixture. After 5 min of stirring, 60 μL of $NH_3 \cdot H_2O$ was added into the mixture to trigger the polymerization reaction. The reaction was allowed to stir for 24 h at 25 ° C to obtain $RuSiO_2$ nanoparticles. Then, 2 mL of acetone were added into the mixture and the solution were sonicated for 10 min to break the emulsion. The precipitation was washed three times with ethanol and once with distilled water to remove the residual surfactant molecules and extra $Ru(bpy)_3^{2+}$. Finally, $RuSiO_2$ was dispersed with ethanol to a final volume of 4 mL. One milliliter (1 mL) of the above $RuSiO_2$ nanoparticles suspension (4.4 mg/mL) was diluted with ethanol to 5 mL, followed by the addition of 400 μL of APTES. After being stirred for 8 h at 30 °C, the mixture was centrifuged and washed two times with ethanol and two times with distilled water to remove the excess APTES. The aminoterminated $RuSiO_2$ nanoparticles then were obtained.

Cell culture

The reagents for cells experiments were all supported by Nanjing KeyGen Biotech. Co.; Ltd. (China). The human breast cancer cells(MCF-7) were cultured with DMEM medium supplemented with 10% fetal calf serum, 4.5 $g \cdot L^{-1}$ glucose, 0.584 $g \cdot L^{-1}$ L-glutamine, 3.7 $g \cdot L^{-1}$ sodium bicarbonate, 0.11 $g \cdot L^{-1}$ sodium pyruvate, 80 $U \cdot mL^{-1}$ penicillin and 80 $mg \cdot mL^{-1}$ streptomycin. Cell culture environment was maintained at 37 °C under a humidified atmosphere containing 5% CO_2. Cells in exponential growth phase were digested for 5 min by trypsin (0.1%, m/v). These cells were collected and separated from the medium through centrifugation at 1000 rpm for 5min, and then washed three times with sterile phosphate buffer solution (PBS, 10 mM, pH 7.4).

Preparation of HA-$RuSiO_2$ and EGF-g-C_3N_4

ECL probes HA-$RuSiO_2$ and EGF-g-C_3N_4 were prepared as following procedures. For EGF-g-C_3N_4, first, the carboxylic acid groups on the surface of g-C_3N_4 nanosheets (30 mg·mL^{-1}, 1 mL) were activated by a mixture containing 20 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 10 mM N-hydroxysuccinimide (NHS) for 1 h at room temperature. Then, 100 μL of 50 ng·mL^{-1} EGF was reacted with the activated g-C_3N_4 at 37 °C for 12 h. Next, the precipitum was centrifuged at 10000 rpm for 10 min and then washed with deionized water. Finally, the formed EGF associated g-C_3N_4 nanosheets was diluted to 1.0 mL with PBS solution (10 mM, pH 7.4) and stored at 4 °C. HA-$RuSiO_2$ was prepared by adding 1 mL of the as-prepared aminoterminated $RuSiO_2$ nanoparticles to 1 mL of a 1 mM HA mixture containing 20 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 10 mM N-hydroxysuccinimide (NHS), and this mixture was vibrated softly for 6 h at room temperature. The resulted HA-$RuSiO_2$ was dispersed in 1.0 mL of PBS solution (10 mM, pH 7.4) and stored at 4 °C.

Fabrication of cytosensor

Folic acid (FA) was activated in the activation solution containing 20 mM EDC and 10 mM NHS overnight. Afterward, the activated FA was dropped on GCE/PAniH and incubated for 4 h. Unconjugated FA was washed away from the electrode surface with PBS. MCF-7 cells were treated by 4% paraformaldehyde (PFA) and then incubated with HA-$RuSiO_2$ (group C), EGF-g-C_3N_4 (group A) and the two ECL probes (HA-$RuSiO_2$ and EGF-g-C_3N_4, group B) solution for 30 min at room temperature, respectively. Three GCE/PAniH/FA electrodes were introduced to construct the spatially separated ECL cytosensor. The cell suspension of group C, group A was dropped on GCE_C/PAniH/FA, GCE_A/PAniH/FA, respectively and then incubated at 37 °C for 1 h. Lu-Au NPs was firstly dropped on GCE_B/PAniH and then FA was introduced. After that, cell suspension of group B was dropped on the electrode. In this step, the cells were captured on PAniH surface through the specific interaction between FA on electrode and FA receptors on MCF-7 surface. The above three electrodes was denoted as WE 3, WE 1 and WE 2, respectively. Finally, WE 3, WE 1 and WE 2 were placed in three independent electrolysis bathes containing 0.1 M $K_2S_2O_8$, 0.01 M PBS and 0.1 M TPA solution, respectively and scanned from 1.8 V to -1.8 V under the scan rate of 0.1 V·s^{-1}. The photomultiplier tube voltage was set at 600 V.

Section 2: UV-Vis absorption spectra of ECL probes

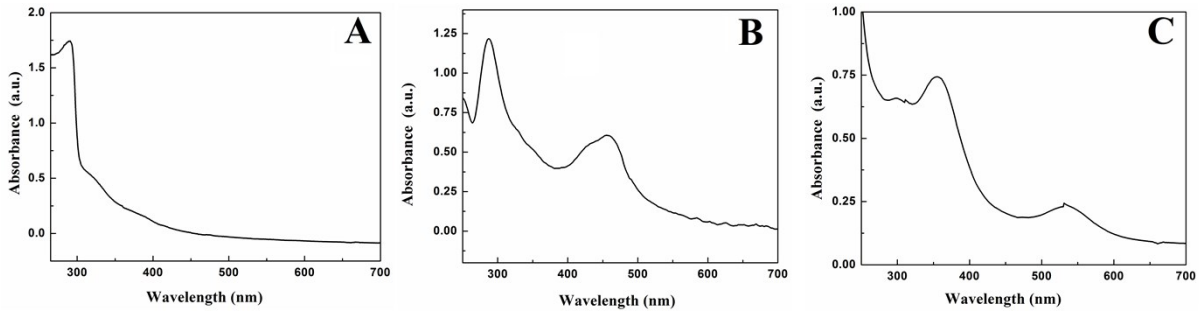

Fig. S1 UV-Vis absorption spectra of g-C_3N_4 nanosheets (A), RuSiO_2 NPs (B) and Lu-Au NPs (C).

Section 3: Effect of photomultiplier tube voltage (PMT)

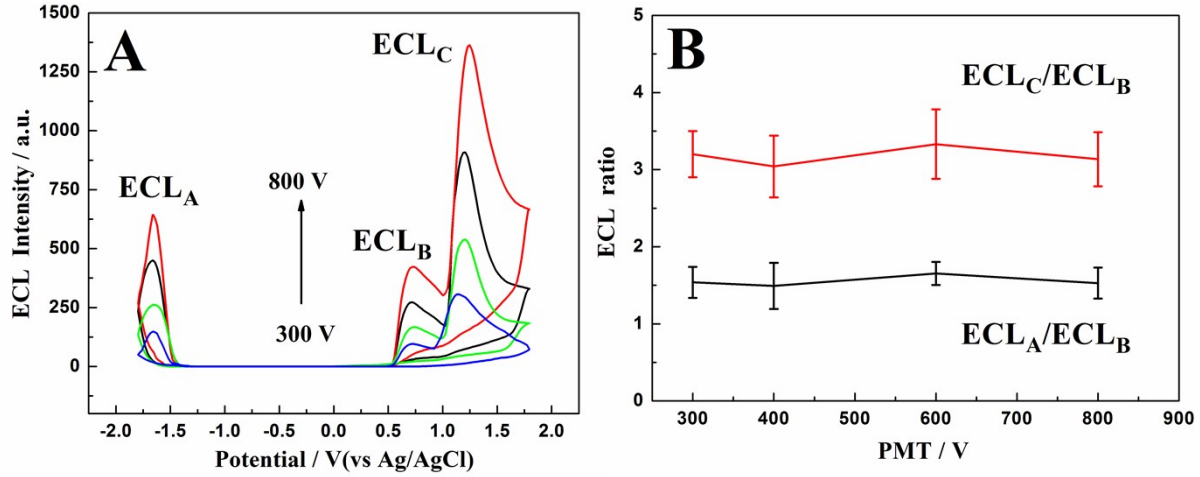

Fig. S2 ECL curves of g-C_3N_4 nanosheets, RuSiO_2 NPs and Lu-Au NPs with different photomultiplier tube voltages (blue: 300 V; green: 400 V; black: 600 V; red: 800 V) with MCF-7 cell concentrations of 5000 cells. (C) The ECL ratio of RuSiO_2 to Lu-Au (ECL_A/ECL_B)and g-C_3N_4 to Lu-Au (ECL_C/ECL_B). ECL_A, ECL_B and ECL_C were obtained in the solution containing 0.1 M $K_2S_2O_8$, 0.01 M PBS and 0.1 M TPA, respectively.

Section 4: Optimization of experimental conditions.

To optimize the performance of ECL cytosensor, several main parameters were investigated. HA-

$RuSiO_2$ and EGF-g-C_3N_4 were centrifuged and the precipitate was diluted to 1.0 mL with PBS, respectively. Afterward, a certain volume of HA-$RuSiO_2$ and EGF-g-C_3N_4 probes were incubated with MCF-7 cells (5.0 × 10^3 cells·mL^{-1}, 0.5 mL), respectively and then diluted to 1.0 mL with PBS. GCE_A/PAniH/FA and GCE_C/PAniH/FA were employed to capture cells and finally tested in $K_2S_2O_8$ and TPA, respectively. The ECL intensity increases with the dosage of HA-$RuSiO_2$ and EGF-g-C_3N_4 increasing to 70 μL and 100 μL, respectively and then both tend to level off (Fig. S3A and B), suggesting 70 μL of HA-$RuSiO_2$ and 100 μL of EGF-g-C_3N_4 can adequately combine with the CD44 and EGFR on cell surface, respectively. The capture time of cells is another important parameter. As the cell capture time progressively increases, as shown in Fig. S3C and D, the ECL intensity of HA-$RuSiO_2$ and EGF-g-C_3N_4 increase and then tend to level off after 60 min, indicating that the optimum capture time is 60 min. These optimal experiment parameters are used in the further measurements.

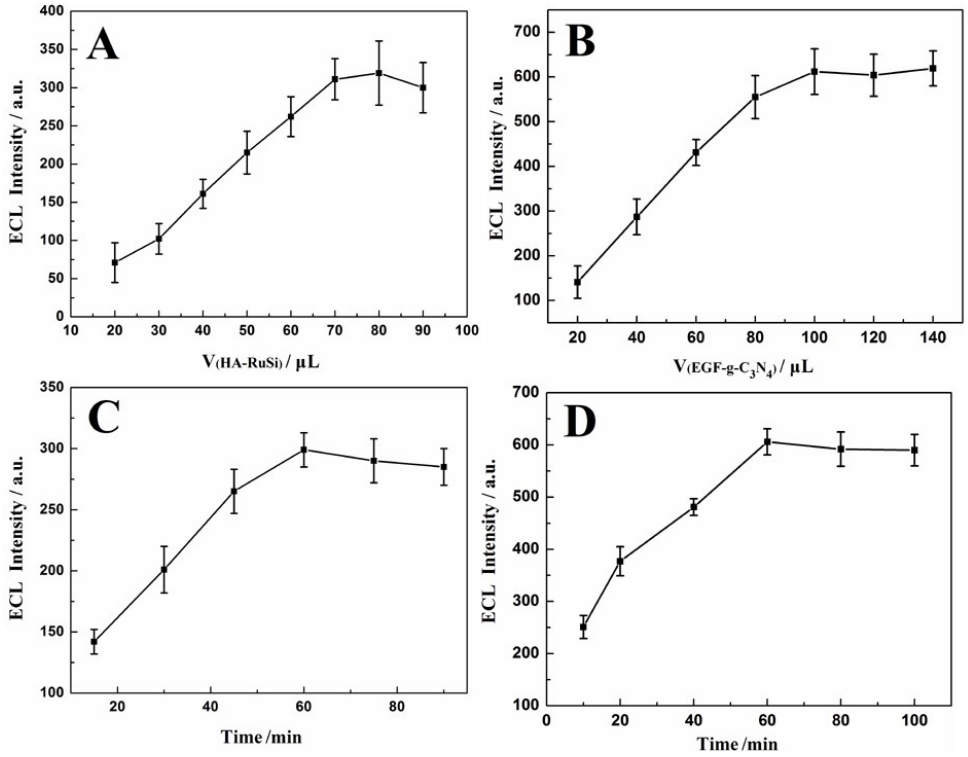

Fig. S3 Dependence of ECL intensity for different volume of HA-$RuSiO_2$ and (A) and EGF-g-C_3N_4 (B) incubated with cells; Dependence of ECL intensity for HA-$RuSiO_2$ (C) and EGF-g-C_3N_4 (D) on the capture time of cells.

Section 5: Quantitative Detection of Cell

Table S1 Determination of MCF-7 cells

Linear regression equation	Linear range (cells mL^{-1})	Correlation coefficient	Detection limit (cells mL^{-1})
$ECL_A/ECL_B = (7.943 \times 10^{-5}) C_{cell} + 0.5429$	$5.0 \times 10^2 \sim 2.5 \times 10^4$	0.9803	200
$ECL_C/ECL_B = (9.391 \times 10^{-4}) C_{cell} + 0.1953$	$5.0 \times 10^2 \sim 5.0 \times 10^4$	0.9884	200

Section 6: Specificity of the cytosensor

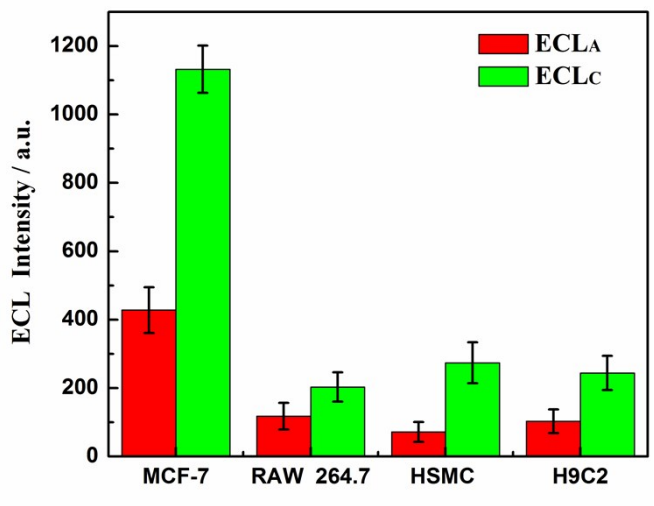

Fig. S4 Specificity of the detection of MCF-7 against potentially interference cells.

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