Electronic Supplementary Information

Bio-bar-code dendrimer-like DNA as signal amplifier for cancerous cells assay using ruthenium nanoparticle-based ultrasensitive chemiluminescence detection

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**Chemicals.** The oligonucleotides sequences (**Table S1**) were commercially synthesized and PAGE purified by SBS Genetech Co. Ltd. (China). RuCl₃·3H₂O was purchased from Shanghai Zenith Company (China). A luminol (standard powder, Sigma-Aldrich) stock solution (1.0 × 10⁻² M) was prepared by dissolution in 0.1 M NaOH and further stored in dark. The stock solution was consecutively diluted with 0.02 M NaOH-NaHCO₃ in order to obtain the proper solution used for FI-CL determination. T4 DNA ligase was ordered from Beijing TransGen Biotech (China). N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) were purchased from Sigma. Double-distilled, deionized water was used throughout the experiments. Whole blood samples were provided by Qingdao Papermaking Hospital.

**Table S1. DNA Sequences Used in This Work**

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**Apparatus.** The CL detection was conducted on a flow injection chemiluminescence (FI-CL) instrument (MPI-F, Remex Analytical Instrument Co. Ltd., Xi’an, China), including a model
IFIS-D flow injection system, a model RFL-1 luminometer, and a computer. The kinetics of CL signals after adding quenchers to luminol-\( \text{H}_2\text{O}_2\)-Ru\(^{3+}\) system were investigated on a BPCL ultraweak luminescence analyzer (Institute of Biophysics Academic Sinica, Beijing, China). UV-vis and fluorescence spectra were recorded on a Cary 50 UV-Vis-NIR spectrophotometer (Varian, USA) and a F-4500 fluorescence spectrophotometer (HITACHI, Japan), respectively. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) images were taken with a H-7650 (HITACHI, Japan) and a JSM-6700F microscope (HITACHI, Japan), respectively.

**Preparation of Ruthenium Nanoparticles (RuNPs).** RuNPs were prepared according to the Viau’s protocol through the reduction of ruthenium (III) chloride in a liquid polyol. Briefly, RuCl\(_3\)-3H\(_2\)O (0.32 mmol) and sodium acetate (1 mmol) were dissolved in 100 ml of 1,2-propanediol. When the temperature was reached at 150 °C for 10 min with stirring, the color of the solution turned from intense red to pale green and finally to yellowish brown, indicating the reduction and formation of RuNPs. After cooling down the resulting colloidal suspension to room temperature with stirring, the colloidal RuNPs were obtained and separated by centrifugation at 12,000 rpm for 30 min. The soft sediment was resuspended in 0.01 M PBS solution and stored at 4 °C for further use. A UV-vis spectrum was also recorded to confirm the formation of Ru-NPs (Fig. S1A), and SEM were recorded to confirm the average size of Ru-NPs (Fig. S1B).

**Construction of Y-DNA and bbc-DL-DNA.** Stock solutions of oligonucleotides were prepared by dissolving in annealing buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 50 mMNaCl). Taking the construction of Y\(_0\)-DNA (shorten as Y\(_0\)) as example, the same molar amount of Y\(_{0a}\), Y\(_{0b}\) and Y\(_{0c}\) were mixed with a final concentration of 5 mM for each
oligonucleotide. After denaturing at 95 °C for 2 min, the solution was quickly cooled down to 60 °C, and further annealing to 4 °C with a continuous temperature decrease at a ratio of 2 °C/min. Similarly, Y₁, and Yₙ and so on were constructed according to above procedures.

For the construction of bbc-DL-DNA, individual Y-DNA was ligated specifically to corresponding Y-DNA. Briefly, 0 generation of DL-DNA (shorten as G₀) was actually the structure of Y₀; G₁ was fabricated by ligating 3 Y₁ with 1G₀; G₂ was fabricated by ligating 6 Y₂ with 1G₁. Other higher generations of DL-DNA were fabricated in the same way. Note that, for the construction of functionalized G₄, two kinds of Y₄ (Y₄a + Y₄b + Y₄c → Y₄ and Y₄a + Y₄b + Y₄l → Y₄', l standing for linker DNA, Y₄b and Y₄c were modified with amino-group) were added at the molar ratio of 23:1 to perform the concept of bio-bar-code DL-DNA (bbc-DL-DNA). For each ligation, the Y-DNA monomer was ligated in ligase buffer containing T4 DNA ligase (200 units/µL) at 25 °C for 1 h.

**Agarose Gel Electrophoresis.** Each generation DL-DNA samples were characterized on agarose Ready-Gel. Agarose gel (3%) were prepared in Tris-acetate-EDTA (TAE) buffer (40 mM Tris,20 mM acetic acid and 2 mM EDTA, pH 8.5) and run at 100 V for 30 min. The gel was stained with ethidium bromide (0.5 μg/ ml) in TAE solution. The visualization and photography were performed using a digital camera under UV illumination. The results are shown in Fig. 1 in the main text.

**Fabrication of RuNP-bbc-DL-DNA/aptamer-MBs Conjugates.** A suspension of carboxylated MBs (100 µL) was previously washed with 400 µL of 0.1 M imidazol-HCl buffer (pH 7.0) three times, and activated in a 0.2 M NHS solution (200 µL) and a 0.8 M EDC solution (200 µL) at 37 °C for 30 min, followed by washing three times with 400 µL of 0.01 M PBS buffer.
and resuspended to a final volume of 200 µL. And then, 500 µL of 2.0 × 10⁻⁸ M amino-modified aptamer was added to ~50 µL of the above resulting MBs solution, and incubated at 37 °C for 8 h with gentle shaking. Excess aptamers were removed by magnetic force, followed by washing the resulting aptamer-MBs three times with PBS buffer. The RuNP-bbc-DL-DNA/aptamer-MBs conjugates were fabricated by adding 200 µL of G₄ bbc-DL-DNA solution to the above prepared aptamer-MBs and incubated at 37 °C for 1 h, followed by reacted with Ru-NPs solution (1.0 × 10⁻⁴ M) for 12 h. The resulting RuNP-bbc-DL-DNA/aptamer-MBs conjugates were separated from the excess unlabeled Ru-NPs solution magnetically, washed three times with 200 µL of PBS buffer, and resuspended in 200 µL PBS buffer for further use at 4 °C.

**Cancer Cell Culture.** Ramos cells (target cells) and CEM cells (control cells) were cultured in cell flasks separately in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 100 IU/mL penicillin-streptomycin in a humidified atmosphere with 5% CO₂ at 37 °C. Prior to the experiments, the cell density was counted using a hemocytometer. And then, a 1.0 mL of ~8.0 × 10⁶ cells suspension was centrifuged at 3500 rpm for 5 min and washed five times with phosphate-buffered saline (18.6 mM phosphate, 4.2 mM KCl, and 154.0 mM NaCl). The resulting cells suspension was resuspended in 1.0 mL cell media buffer.

**Magnetic Extraction.** After adding 50 µL of RuNP-bbc-DL-DNA/aptamer-MBs conjugates to each cell sample followed by incubating for 15 min at 37 °C, a magnetic field was employed to separate the supernatant which contained the released RuNP-bbc-DL-DNA probes resulting from the structure-switching of cell aptamers. The amount of released RuNP-bbc-DL-DNA probes were proportional to the amount of target cells and decanted using a pipette for the following FI-CL detection.
**FI-CL Detection.** 200 μL of 0.2 M nitric acid solution was used to dissolve RuNPs which were labeled on the released RuNP-bbc-DL-DNA probes. After adjusting the pH of resulting Ru\(^{3+}\) solution to 5.0 with NaOH, the volume of the solution was adjusted to 5 mL with 0.02 M HNO\(_3\) (pH 5.0). After optimizing the luminol-H\(_2\)O\(_2\)-Ru\(^{3+}\) CL system, the FI-CL detection was performed by mixing 5.0 × 10\(^{-4}\) M luminol in pH 10.0, 0.02 M NaOH-NaHCO\(_3\) buffer solution with 4.0 × 10\(^{-2}\) M H\(_2\)O\(_2\), and then reacting with metal ions in the flow cell to produce CL signal.
Characterization of Ruthenium Nanoparticles (RuNPs) and Its Assembly on MBs. The synthesized RuNPs were characterized by UV-vis spectra and SEM images. From Fig. S1A, the formation of RuNPs could be confirmed when the UV-vis absorption band at ~400 nm disappeared, indicating that the reduction of Ru$^{3+}$ salt was complete. The size distribution of RuNPs was characterized by SEM images. From Fig. S1B, by reduction of RuCl$_3$ in 1,2-propane diol, a mean diameter of 4 nm-sized RuNPs were obtained. In addition, SEM observation of RuNPs prepared by this method exhibited a good dispersion without agglomeration, which further showed an excellent stability against agglomeration since no evident precipitation of metal powder was observed upon standing the colloidal solution at room temperature for one week.

Fig. S1. (A) UV-vis absorption spectra of RuCl$_3$ solution (black curve) and RuNPs (red curve). (B) The SEM images of RuNPs with an average diameter of 4 nm.

The TEM images of magnetic beads (MBs) before and after conjugation with DL-DNA capped with RuNPs were recorded and shown in Fig. S2. The TEM of the construction of RuNP-bbc-DL-DNA/aptamer-MBs conjugates were shown in Fig. S2B. Numerous RuNPs could be seen on the surface of the MB compared to that of MB before conjugation as shown in Fig.
S2A. The results indicated that the conjugates of RuNP-bbc-DL-DNA/aptamer-MBs were constructed satisfactorily as expectation.

Fig. S2 The TEM images of magnetic beads (MBs) (A) before and (B) after conjugation with DL-DNA capped with RuNPs.

Moreover, the amount of RuNPs constructed on the surface of one MB was determined as follows:

**Preparation of FI-CL calibration curve of standard RuNPs solutions.** Standard RuNPs solutions were prepared from the solution of $1.0 \times 10^{-3}$ M RuNPs. The FI-CL calibration curve of RuNPs is shown in Fig. S3, the regression equation could be expressed as $Y = -0.0596X^2 + 17.4568X + 31.3619$ (X is the concentration of RuNPs solution, $10^{-5}$ M; Y is the FI-CL intensity, n = 12, R = 0.9980).

Fig. S3 The calibration curve of peak height versus the concentration of RuNPs solution from $1.0 \times 10^{-5}$ to $1.0 \times 10^{-3}$ M.
Determination of the surface coverages of RuNPs on MBs. A $1.0 \times 10^{-3}$ M RuNPs solution was reacted with DL-DNA/aptamer-MBs conjugates and incubated for 12 h. Then the supernatant was taken for FI-CL detection. The number of RuNPs immobilized on the MBs can be quantitatively calculated by the FI-CL intensities differences between the RuNPs solution before and after immobilization, which is calculated as below.

- FI-CL intensity of the background of luminol-H$_2$O$_2$ FI-CL system: 63
- FI-CL intensity of the supernatant before immobilization: 1242
- → Concentration of RuNPs before immobilization: $9.98 \times 10^{-4}$ M
- FI-CL intensity of the supernatant after immobilization: 1027
- → Concentration of RuNPs after immobilization: $7.04 \times 10^{-4}$ M
- Thus, the concentration of RuNPs immobilized on MBs: $(9.98 - 7.04) \times 10^{-4} = 2.94 \times 10^{-4}$ M

Calculation of moles of MBs in a given preparation

- MNP diameter = $0.75 \times 10^{-4}$ cm
- MNP Volume = $4/3\pi r^3 = 1.77 \times 10^{-12}$ cm$^3$
- Mass MNP = $\rho_{\text{MNP}} \times V_{\text{MNP}} = 1.18 \times 1.77 \times 10^{-12} = 2.09 \times 10^{-12}$ (g / MNP)
- The concentration of MNP in 0.01g/mL MNP solution for the preparing
- $(0.01 \times 10^3) / (2.09 \times 10^{-12}) / (6.02 \times 10^{23}) = 7.95 \times 10^{-12}$ M
- Thus, $(2.94 \times 10^{-4}) / (7.95 \times 10^{-12}) = 3.7 \times 10^7$ RuNPs were constructed on the surface of one MB.
Optimization of Luminol-H$_2$O$_2$-Ru$^{3+}$ FI-CL System. The effects of luminol and H$_2$O$_2$ concentrations, and Ru$^{3+}$ standard solution pH in luminol-H$_2$O$_2$-Ru$^{3+}$ FI-CL system were investigated comprehensively. As shown in Fig. S4A, the signal/background ratios increased with an increase in luminol concentrations from $1.0 \times 10^{-4}$ to $5.0 \times 10^{-4}$ mol/L, and decreased with further increasing luminol concentrations. Furthermore, the effect of H$_2$O$_2$ concentration on luminol-H$_2$O$_2$-Ru$^{3+}$ CL system was studied in the range of $1.0 \times 10^{-3}$ ~ $1.0 \times 10^{-1}$ mol/L. As shown in Fig. S4B, the CL signal/background ratios increased with increasing H$_2$O$_2$ concentrations in the range of $1.0 \times 10^{-3}$ ~ $4.0 \times 10^{-2}$ mol/L, and a maximal signal was obtained at $4.0 \times 10^{-2}$ mol/L. Therefore, $5.0 \times 10^{-4}$ mol/L and $4.0 \times 10^{-2}$ mol/L were selected as the optimal concentrations of luminol and H$_2$O$_2$, respectively.

As a critical factor of luminol-H$_2$O$_2$-Ru$^{3+}$ CL reaction system, the pH value of Ru$^{3+}$ standard solution was studied extensively through adjusting the pH values of Ru$^{3+}$ standard solution in the range of 2.5 to 7.0. From Fig. S4C, the maximum CL signal/background ratio was occurred at pH 5.0 of Ru$^{3+}$ standard solution.

After optimizing the conditions of luminol-H$_2$O$_2$-Ru$^{3+}$ FI-CL system, the calibration curve of relative CL intensities versus Ru$^{3+}$ concentrations was further investigated. As shown in Fig. S4D, the CL intensities were found to increase gradually with increasing the concentration of Ru$^{3+}$. For the concentrations of $1.0 \times 10^{-8}$ to $1.0 \times 10^{-6}$ g/mL Ru$^{3+}$ standard solution, the nonlinear function could be expressed as $I = -0.0708C^2 + 17.7950C + 34.6885$ (I is the relative CL intensity; C is the concentration of Ru$^{3+}$, $10^{-8}$ M; $n = 12$, $R^2 = 0.9978$); for the concentrations of $1.0 \times 10^{-8}$ to $1.0 \times 10^{-7}$ g/mL Ru$^{3+}$ standard solution, the linear regression equation could be expressed as $I =$.
22.0154C + 13.5034 (C is the concentration of Ru$^{3+}$, 10$^{-8}$ g/mL; I is relative the CL intensity, n = 7, 
R = 0.9961).

Fig. S4 Effects of the reactant conditions on the luminol-H$_2$O$_2$-Ru$^{3+}$ CL system. (A) Effects of the 
concentration of luminol: pH 10.0, 0.02 M NaOH-NaHCO$_3$ buffer solution, 7.5 × 10$^{-3}$ M H$_2$O$_2$
and pH 5.0, 5.0 × 10$^{-7}$ g/mL Ru$^{3+}$ solution. (B) Effects of the concentration of H$_2$O$_2$: 5.0 × 10$^{-4}$ M 
luminol in pH 10.0, 0.02 M NaOH-NaHCO$_3$ buffer solution, pH 5.0, 5.0 × 10$^{-7}$ g/mL Ru$^{3+}$
solution. (C) Effects of Ru$^{3+}$ solution pH: 5.0 × 10$^{-4}$ M luminol in pH 10.0, 0.02 M 
NaOH-NaHCO$_3$ buffer solution, 4.0 × 10$^{-2}$ M H$_2$O$_2$ and 5.0 × 10$^{-7}$ g/mL Ru$^{3+}$ solution. (D) CL 
signal calibration curve of Ru$^{3+}$ standard solution. The concentration of luminol and H$_2$O$_2$ were 
5.0 × 10$^{-4}$ M in pH 10.0, 0.02 M NaOH-NaHCO$_3$ buffer solution and 4.0 × 10$^{-2}$ M, respectively. 
The pH of Ru$^{3+}$ standard solution was 5.0.
Investigation on the Mechanism of Luminol-H$_2$O$_2$-Ru$^{3+}$ CL System.

Fig. S5 (A) The UV absorption spectra of luminol (black), luminol-H$_2$O$_2$ (red), luminol-H$_2$O$_2$-Ru$^{3+}$ (1.0 × 10$^{-6}$ g/mL, green), luminol-H$_2$O$_2$-Ru$^{3+}$ (1.0 × 10$^{-7}$ g/mL, blue), luminol-H$_2$O$_2$-Ru$^{3+}$ (1.0 × 10$^{-8}$ g/mL, cyan), luminol-Ru$^{3+}$ (1.0 × 10$^{-6}$ g/mL, magenta), luminol-Ru$^{3+}$ (1.0 × 10$^{-7}$ g/mL, yellow) luminol-Ru$^{3+}$ (1.0 × 10$^{-8}$ g/mL, dark yellow). (B) Fluorescence spectra of luminol (black), luminol-Ru$^{3+}$ (1.0 × 10$^{-8}$ g/mL, red), luminol-Ru$^{3+}$ (1.0 × 10$^{-7}$ g/mL, green), luminol-Ru$^{3+}$ (1.0 × 10$^{-6}$ g/mL, blue), luminol-H$_2$O$_2$-Ru$^{3+}$ (1.0 × 10$^{-8}$ g/mL, cyan) luminol-H$_2$O$_2$-Ru$^{3+}$ (1.0 × 10$^{-7}$ g/mL, magenta) luminol-H$_2$O$_2$-Ru$^{3+}$ (1.0 × 10$^{-6}$ g/mL, yellow). (C) Effect of quenchers on the CL mechanism of luminol-H$_2$O$_2$-Ru$^{3+}$ system. Luminol, H$_2$O$_2$ and 1.0 × 10$^{-7}$ g/mL Ru$^{3+}$ were mixed and reacted with H$_2$O (black), 100 µg/mL SOD (red), 0.01 M methyl benzoate (green), and 0.01 M mannitol (blue), respectively. The concentrations of luminol and H$_2$O$_2$ were 5.0 × 10$^{-4}$ M and 4.0 × 10$^{-2}$ M, respectively. (D) The CL mechanism of luminol-H$_2$O$_2$-Ru$^{3+}$ system.
In order to prove whether the mechanism of luminol-H$_2$O$_2$-Ru$^{3+}$ system was the “coordination complex mechanism” or not, the UV-vis and fluorescence spectra were first investigated. From Fig. S5A and B, the UV-vis absorption spectra and fluorescence spectra of luminol, luminol-H$_2$O$_2$, luminol-Ru$^{3+}$ and luminol-H$_2$O$_2$-Ru$^{3+}$ with different Ru$^{3+}$ concentrations seem nearly identical. Thus, it could be proposed that there was no coordination complex formed during the reaction, and the possible CL mechanism of luminol-H$_2$O$_2$-Ru$^{3+}$ system was not the “coordination complex mechanism”. Consequently, we further investigated the possible CL mechanism of luminol-H$_2$O$_2$-Ru$^{3+}$ system by adding different CL quenchers, such as ·O$_2^-$ quencher, superoxide dimutase (SOD), and HO· quenchers, mannitol and methanol. From Fig. S5C of CL kinetics, the CL intensities were obviously decreased by HO· quenchers, especially quenched by ·O$_2^-$ quencher. Thus, it is reasonable that the possible CL mechanism of luminol-H$_2$O$_2$-Ru$^{3+}$ system was the “free radical mechanism”, which is summerized in Fig. S5D.
Control Experiment by Employing Single RuNP as Labels for Assay.

Fig. S6 Schematic illustration of employing single RuNP as labels for the assay of cancer cells based on luminol-H$_2$O$_2$-Ru$^{3+}$ FI-CL reaction system.

As controls, we also investigated the assay by employing single RuNP as labels for cancer cells detection (Fig. S6). The procedures were performed as described in the main text except using single RuNP rather than bbc-DL-DNA as labels at step (II). As shown in Fig. S7, under the optimized experimental conditions, the FI-CL signals of luminol-H$_2$O$_2$-Ru$^{3+}$ reaction system increased with the increase of concentrations of Ramos cells ranging from 5000 to 100000 cell/mL with a nonlinear function of $I = -3.4372 \times 10^{-8}C^2 + 0.0117C - 6.0871$ ($I$ is the FI-CL intensity; $C$ is the concentration of target cells, $n = 9$, $R^2 = 0.9991$) and detection limit of 3622 cells/mL, which was ~50-fold higher than that obtained by employing bbc-DL-DNA as labels.

Fig. S7 The calibration curve of peak heights versus the concentrations of target cells from 5000 to 100000 cells/mL.
Investigation of the impact of the DL-DNA capped with RuNPs on the sensitivity of this approach. The impact of the DL-DNA capped with RuNPs on the sensitivity of this approach has been investigated (Fig. S8). Upon the introduction of cell samples, bbc-DL-DNA labels (step IVa) and RuNP-bbc-DL-DNA labels (step IVb) were released from the surface of MBs and following dissolved via nitric acid for luminol-H$_2$O$_2$ CL system. From the results of our study, the CL intensities obtained from employing bbc-DL-DNA as labels without incubating with RuNPs ($h_{\nu_1}$) were approximate to that of luminol-H$_2$O$_2$ system ($h_{\nu_0}$), while significantly increased by employing RuNPs-bbc-DL-DNA as labels ($h_{\nu_2}$). The concentration of target cells for each assay was 1000 cells/mL. Thus, it could be deduced that the DL-DNA has no influence on the sensitivity of this approach, which could ascribe to the denaturation of DNA dendrimers under the strong acid conditions at step V in order to dissolve RuNPs into Ru$^{3+}$ ions for FI-CL detection.

Fig. 8 Investigation of the impact of the DL-DNA capped with RuNPs on the sensitivity of this approach.
1 Mixed Cell Samples Assay.

2

3

4 Fig. S9 FI-CL signals of (a) blank sample, (b) pure Romas target cells sample, and (c) Romas target cells and CEM control cells mixing sample. Both concentrations of target cells and control cells are 2000 cells/mL.

5

6 Table S2. FI-CL Signals of Blood Samples Spiked with and without Romas Target Cells

7

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8 [a] No FI-CL response