Supporting Information

Potential-Resolved Electrochemiluminescence for Simultaneous Determination of Multiplex Bladder Cancer Markers

Junke Wang[a], Fenglian Guo[a], Jingjing Zhang[a]*, Jie Chao[a,b]*

[a]State Key Laboratory of Organic Electronics and Information Displays & Jiangsu Key Laboratory for Biosensors, Institute of Advanced Materials (IAM), Jiangsu National Synergetic Innovation Center for Advanced Materials (SICAM), Nanjing University of Posts and Telecommunications, 9 Wenyuan Road, Nanjing 210023, China.

[b]Portland Institute, Nanjing University of Posts and Telecommunications, Nanjing 210023, China

*Corresponding authors:

E-mail addresses: jingjingzhang@njupt.edu.cn (J. J. Zhang); iamjchao@njupt.edu.cn (J. Chao)

Table of Contents for Supporting Information

1. Reagents

$HAuCl_4 \cdot 3H_2O$ (hydrogen tetrachloroaurate trihydrate), Tris(2,2'-bipyridine)ruthenium dichloride ($Ru(bpy)_3^{2+}$) and sodium borohydride ($NaBH_4$) were bought from Sigma-Aldrich; Zirconyl chloride octahydrate ($ZrOCl_2 \cdot 8H_2O$), 4,4,4,4-(Porphine-5,10,15,20-tetrayl)tetrakis(benzoic acid) (H_2TCPP), Benzoic acid (BDC) and were purchased from Shanghai Macklin Biochemical Co., Ltd.(Shanghai, China). $AgNO_3$, L-arginine (Arg) were purchased from Sinopharm Chemical Reagent Co. Ltd. (China). 6-aze-2-thiothymine was procured from Alfa Aesar Co., Ltd. (China). Tripropylamine (TPA) was obtained from Shanghai Yien Chemical Technology Co., Ltd (Shanghai, China). Tris-magnesium acetate (TM) buffer, ammonium peroxodisulfate (APS) and orthoboric acid were obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). CFHR1, NUMA and antibodies were obtained from Abcam; Oligonucleotide Sequences were synthesized by Sangon Biotech Company (China). The 0.1 M phosphate-buffered saline (PBS; pH 7.4) composed of K_2HPO_4, NaH_2PO_4, NaCl, and KCl were used as the supporting electrolytes in the ECL measurement process. Millipore water obtained from a Millipore water purification system (resistivity: 18.2 MΩ·cm) was used for all experiments.

2. Apparatus

ECL measurements were performed using the BPCL ultraweak luminescence analyzer purchased from Guangzhou Ultraweak Luminescence Technology. The Cyclic voltammetry (CV) measurement was carried out with a CHI600e instrument (CH Instrument Co., USA). Electrochemical impedance spectroscopy (EIS) spectra were collected on a PARSTAT 2273 potentiostat/galvanostat (Advanced Measurement Technology Inc., USA) applying an AC voltage amplitude of 5 mV in a frequency range from 0.1 Hz to 100 kHz in a 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ containing 0.1 M KCl. TEM characterization was conducted using the Hitachi HT7700 system, operated at 100 kV in bright field mode. PAGE images were recorded on G: BOX F3 Gel Imager System (Synoptics Ltd., UK).

3. Oligonucleotide Sequences

Table S1. Sequence information for the nucleic acids used in this study

Name	Sequences (5' - 3')	Modification
S1	CTCAACTGCCTGGTGATACGAGGATGG GCATGCTCTTCCCGACGGTATTGGACCC TCGCATG	5'NH_2 C6
S2	CGATTACAGCTTGCTACACGATTCA GACTTAGGAATGTTCGACATGCGAG GGTCCAATACCG	5'HS C6
S3	CTACTATGGCGGGTGATAAAACGTG TAGCAAGCTGTAATCGACGGGAAGA GCATGCCCATCC	5'HS C6
S4	TTTATCACCCGCCATAGTAGACGTAT CACCAGGCAGTTGAGACGAACATTC CTAAGTCTGAA	5'HS C6

4. Preparation of TDN-Ab_1

TDN was formed by four precisely designed ssDNA (S1, S2, S3, S4), and sequence information for the nucleic acids used in this study is shown in Table S1. The concentrations of the four ssDNA stock solutions are recalculated using UV-Vis spectroscopy. The four ssDNA solutions were mixed with a molar ratio of 1:1:1:1 (2μL, 50 μM) in 92 μL TM buffer (20 mM Tris-HCl, 50 mM $MgCl_2$, 100 mM KCl, pH 8.0). Then, the mixture was heated at 95 °C for 5 min and quickly cooled to 4 °C on a PCR thermocycle instrument. After completing the self-assembly, the TDN were obtained. Subsequently, 2.5 % glutaraldehyde solution was added to the synthesized TDN and reacted at room temperature for 30 min, then anti-CFHR1 (Ab_1) and anti-NUMA (Ab_1) were added respectively, followed by shaking for 30 min. The resulting solution was

ultrafiltered through an ultrafiltration tube to obtain the capture probes TDN-CFHR1-Ab_1 and TDN-NUMA1-Ab_1 (TDN-Ab_1).

5. Synthesis of Ru-MOF

Ru-MOF composite was prepared according the reported method with slight modification.[1,2] First, $ZrOCl_4 \cdot 8H_2O$ (75 mg), H_2TCPP (25 mg) and BDC (550 mg) were ultrasonically dissolved in 25 mL of N-N′-dimethylformamide (DMF). Then, the mixture was continuously stirred for 5 h at 95 °C, followed by centrifugation and washed with DMF and ethanol, the product was collected. The obtained MOFs were dried for future use or dispersed in ethanol. 10 mL of $Ru(bpy)_3^{2+}$ solutions (3 mg/mL) was mixed with MOFs, and the solution was then stirred for overnight, the Ru-MOFs product was obtained by centrifugation and washing with DMF and ethanol.

6. Synthesis of Ru-MOF@AuNPs

Ru-MOF@AuNPs were synthesized following our previous in-situ method.[1,2] 70 μL of the as-prepared Ru-MOF solution (40 mg/mL) and 100 μL of $HAuCl_4$ (1%) were added into 30 mL H_2O, followed by magnetic stirring for 5 min. 100 μL of freshly prepared $NaBH_4$ (3.8 mg/mL) was fleetly added into the mixture and kept stirring for 10 min. Finally, the Ru-MOF@AuNPs obtained by centrifugation and washed with water.

7. Synthesis of signal probe (Ru-MOF@AuNPs-Ab_2)

The as-prepared Ru-MOF@AuNPs were added into 200 μL of phosphate buffered saline (PBS, pH 7.4). 10 μL of anti-NuMA1 (Ab_2) was added respectively and kept stirred for 2 h to ensure the reaction occurred at room temperature. Thereafter, 100 μL of 1% BSA was added to block the excess binding sites on the surface of AuNPs. After shaking incubation at 37 °C for 30 min, the resulting precipitates were collected by centrifugation and rinsed with PBS. Finally, the resultant signal probe was dispersed with 200 μL of PBS and stored at 4 °C for later use.

8. Synthesis of AuAgNCs

AuAgNCs were prepared according the reported method with slight modification.[2] Adjust the pH of a mixture of 15 mL, 80 mM ATT and 15 mL, 24 mM $HAuCl_4$ to 10 with NaOH, followed by vigorous stirred for 1 h in the dark. Afterwards, ATT-AuNCs were obtained by precipitation with isopropyl alcohol, and dissolved in H_2O to form 15 mg/mL stock solution. Under intense stirring, 1 mL 10 mM $AgNO_3$ solution was added to 1 mL 0.5 M Arg solution to trigger the complexation of silver ion and amino group in Arg via Ag-N covalent bond. Adjust the pH to 10, the solution was incubated at 37 ℃ for 6 h to obtained Arg-Ag NCs. Subsequently, 6 mL of as-prepared Arg-Ag NCs was mixed with 18 mL ATT-Au NCs to react at 37 ◦C for 24 h to generate hydrogen bonding between ATT and Arg. The obtained AuAgNCs precipitation was centrifuged with isopropyl alcohol and re-dispersed in H_2O and stored at 4 °C for further use.

9. Synthesis of signal probe (AuAgNCs-Ab_2)

First, 400 μL of AuAgNCs was dispersed in 200 μL of PBS (pH 7.4) solution. Then, 10 μL of anti-CFHR1 (Ab_2) (0.1 mg/mL) was added into above solution, and reacted at 37◦C for 2 h with stirring. The remaining active sites were blocked by adding 1% BSA into the above solution and incubation at 37 °C for 30 min. Finally, the AuAgNCs-Ab_2 was obtained after centrifugation and washing, and then dispersed in PBS and stored at 4 °C for later use.

10. Preparation of the ECL immunosensor

The fabrication process of the ECL immunosensor is shown in Scheme 1. To obtain a mirror-like surface, the GCE was sequentially polished by 0.05 μm alumina powder and washed. After drying at room temperature, the GCE was immersed in 0.25% $HAuCl_4$ solution for the electrodeposition of the AuNPs layer at a constant potential of -0.2 V for 60 s. Then, 10 μL of 500 nM TDN-Ab_1 mixture (1:1) was coated onto the GCE and incubated at room temperature for 1 h. The remaining active sites on the electrode were blocked using BSA solution for 30 min. For NUMA1 and CFHR1

detection, 10 μL of NUMA1 and CFHR1 solutions with different concentrations were coated onto the decorated electrode, and then incubated for at 37 °C 1 h. Finally, the ECL immunosensor was fabricated completely by incubating the electrode with 10 μL of Ru-MOF@AuNPs-Ab_2 and AuAgNCs-Ab_2 mixture at 37 °C for 1 h. The prepared ECL immunosensor was stored at 4 °C for next use.

11. ECL detection

The fabricated ECL immunosensor was immersed in 0.1 M PBS (pH 7.4) containing 10 mM TPrA and 0.1 M $K_2S_2O_8$ to investigate the ECL signal by ECL analyzer. The potential range was from -2.0 to 1.3 V with a scanning rate of 0.5 V/s. The voltage of the photomultiplier tube was set to -800 V.

12. PL of AuAgNCs

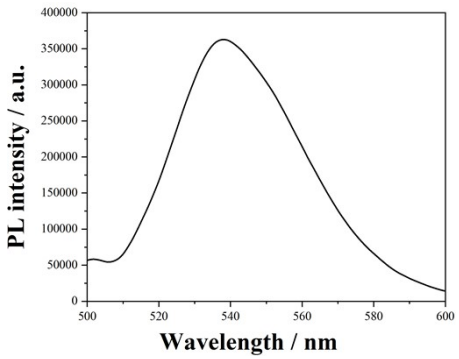

Fig. S1. PL intensity of AuAgNCs.

13. TEM of Ru-MOF

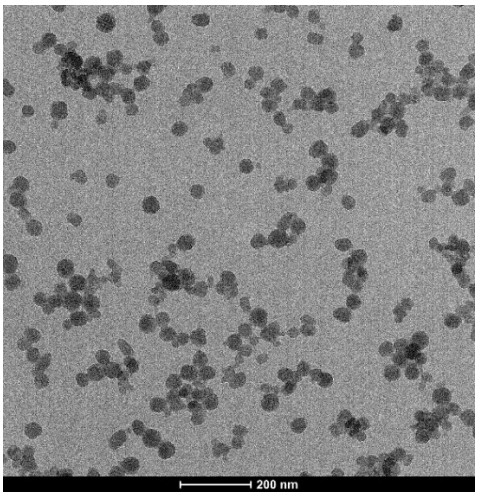

Fig. S2. The TEM of Ru-MOF.

14. HRTEM of Ru-MOF@AuNPs

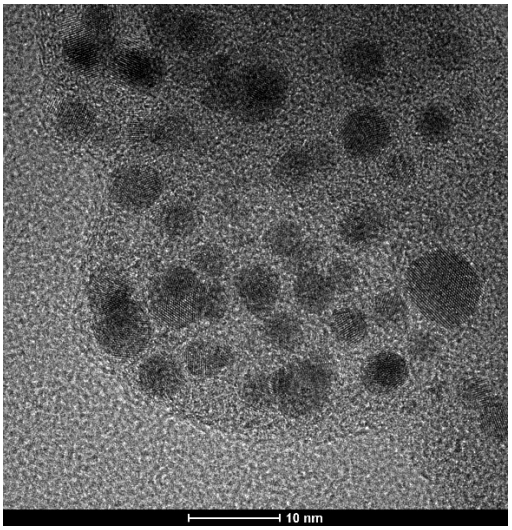

Fig. S3. The HRTEM of Ru-MOF@AuNPs.

15. UV-vis absorption spectra of Ru-MOF@AuNPs

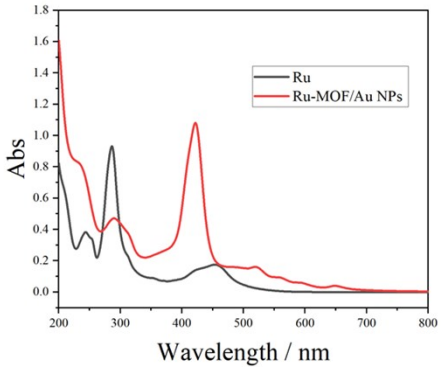

Fig. S4. UV-vis absorption spectra of $Ru(bpy)_3^{2+}$ and Ru-MOF@AuNPs.

16. ECL stability of AuAgNCs and Ru-MOF@AuNPs

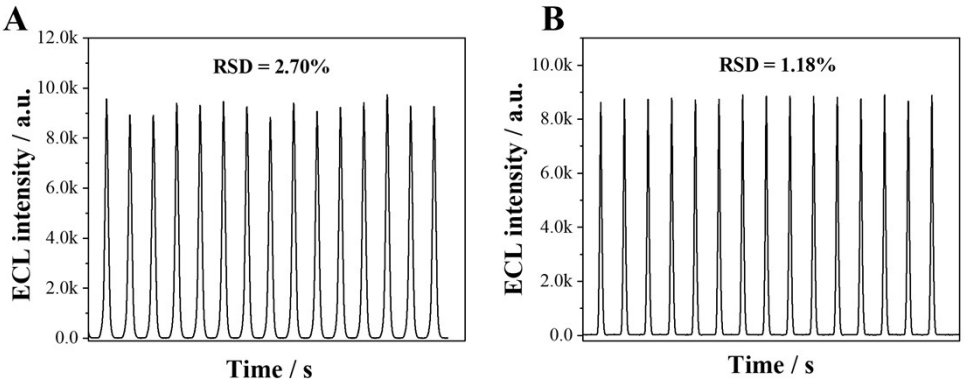

Fig. S5. ECL stability of (A) AuAgNCs and (B) Ru-MOF@AuNPs.

17. CV curves of the Ru-MOF@AuNPs and AuAgNCs in different solution

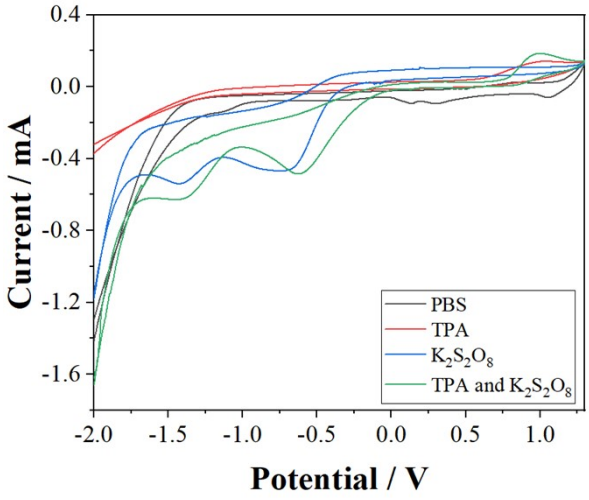

Fig S6. CV curves of the Ru-MOF@AuNPs and AuAgNCs in 0.1 M PBS (black line), 0.1 M PBS containing 10 mM TPA (red line) or 100 mM $K_2S_2O_8$ (blue line), and 0.1 M PBS containing 10 mM TPA and 100 mM $K_2S_2O_8$ (green line). Scan rate: 100 mV/s, scan range: -2.0 - 1.3 V.

The possible mechanisms as follows:

Anode:

$$Ru^{2+}\text{-MOF@AuNPs} \rightarrow Ru^{3+}\text{-MOF@AuNPs} + e^- \quad 1$$

$$Ru^{3+}\text{-MOF@AuNPs} + \text{TPrA} \rightarrow Ru^{2+}\text{-MOF@AuNPs} + \text{TPrA}^{\bullet} \quad 2$$

$$\text{TPA} - e^- \rightarrow \text{TPrA}^{\bullet+} \rightarrow \text{TPrA}^{\bullet} \quad 3$$

$$Ru^{3+}\text{-MOF@AuNPs} + \text{TPrA}^{\bullet} \rightarrow Ru^{2+*}\text{-MOF@AuNPs} \quad 4$$

$$Ru^{2+*}\text{-MOF@AuNPs} \rightarrow Ru^{2+}\text{-MOF@AuNPs} + h\nu \quad 5$$

Cathode:

$$\text{AuAgNCs} + e^- \rightarrow \text{AuAgNCs}^{\bullet-} \quad 1$$

$$S_2O_8^{2-} + e^- \rightarrow SO_4^{2-} + SO_4^{\bullet-} \quad 2$$

$$\text{AuAgNCs}^{\cdot-} + SO_4^{\bullet-} \rightarrow \text{AuAgNCs}^{*} + SO_4^{2-} \quad 3$$

$$\text{AuAgNCs}^{*} \rightarrow \text{AuAgNCs} + h\nu \quad 4$$

18. ECL characterization of the sensor preparation

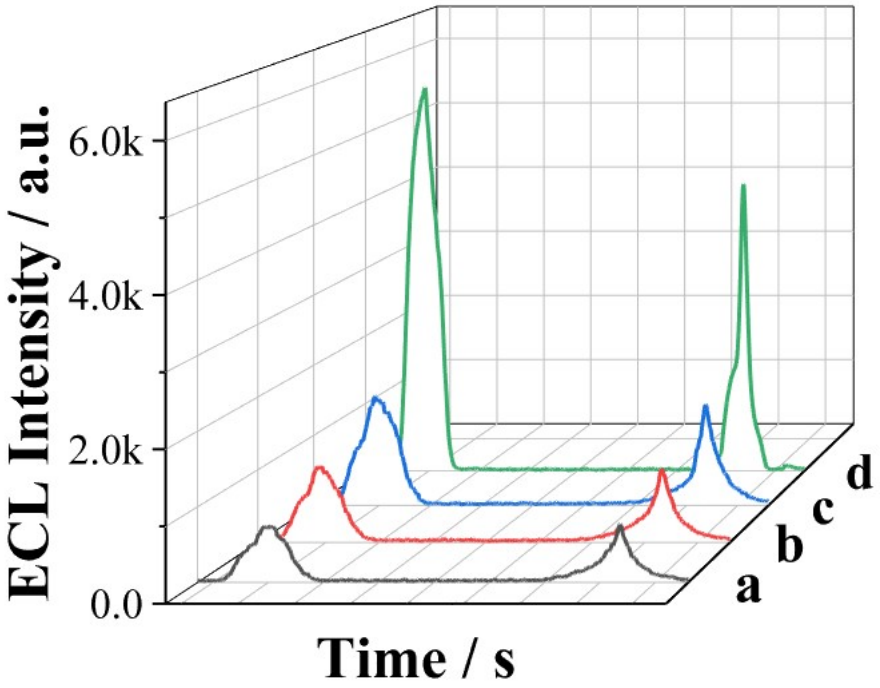

Fig. S7. The ECL intensity of GCE (a), GCE/DpAu/capture probe/BSA/CFHR1 and NUMA1 (b), GCE/DpAu/capture probe/BSA/CFHR1 and NUMA1/Ru-MOF@AuNPs and AuAgNCs (c), GCE/DpAu/capture probe/BSA/CFHR1 and NUMA1/signal probe (d). The concentration of NUMA1 and CFHR1 is 0.05 ng/mL. Working solution: 0.1 M PBS (pH 7.4) containing 10 mM TPrA and 100 mM M $K_2S_2O_8$. Scan rate: 100 mV/s, scan range: -2.0-1.3 V.

19. The optimization data for experimental conditions

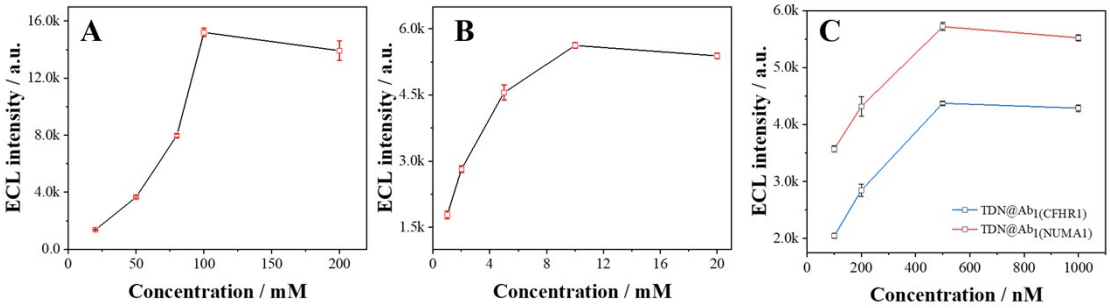

Fig. S8. The effects of the concentrations of $K_2S_2O_8$ (A), TPrA (B) and capture probe (C) on the ECL intensity of the immunosensor were investigated. The error bars represent the standard deviations of three independent measurements. The concentration of NUMA1 and CFHR1 is 0.05 ng/mL.

20. Different methods for NUMA1 and CFHR1 detection.

Table S2. Different Methods for NUMA1 and CFHR1 Detection.

	NUMA1		CFHR1		
Method	Detection range	Detection limit	Detection range	Detection limit	Refs.
EC ELISA	1–100 ng/mL	1.29 ng/mL	1–100 ng/mL	0.97 ng/mL	4
Fluorescence	1-500 pg/mL	0.31 pg/mL	/	/	5
Mass Spectrometry	/	/	/	1.63 μg/mL	6
ECL	0.001-0.5 ng/mL	0.14 pg/mL	0.001-0.5 ng/mL	0.11 pg/mL	This work

21. Recovery experiments for NUMA1 and CFHR1 in human urine samples

Table S3. Analysis of Human Urine Samples

	NUMA1				CFHR1			
Sample number	Added (ng/mL)	Found (ng/mL)	Recovery (%)	RSD (n=3, %)	Added (ng/mL)	Found (ng/mL)	Recovery (%)	RSD (n=3, %)
1	0.005	0.0048	96.0	1.5	0.005	0.0049	98.0	3.3
2	0.01	0.0099	99.0	2.7	0.01	0.0098	98.0	4.6
3	0.05	0.0494	98.8	1.6	0.05	0.0473	94.6	5.8

22. References

1 Z. M. He, X. L. Huang, C. Wang, X. L. Li, Y. J. Liu, Z. J. Zhou, S. Wang, F. W. Zhang, Z. T. Wang, O. Jacobson, J. J. Zhu, G. C. Yu, Y. L. Dai and X. Y. Chen, Angew. Chem. Int. Ed., 2019, 58, 8752-8756.

2 J. J. Zhang, F. L. Guo, J. F. Zhu, Z. M. He, L. Hao, L. X. Weng, L. H. Wang and J. Chao, Anal. Chem., 2023, 95, 11440-11448.

3 Y. Zhou, Y. Zheng, X. Zhu, Y. Chai and R. Yuan, Biosens. Bioelectron., 2021, 190, 113449.

4 S. K. Arya and P. Estrela, Biosens. Bioelectron., 2018, 117, 620-627.

5 W.Y. Zhuge, X.F. Tan, R.Y. Zhang, H. Li and G.X. Zheng, Chinese Chem. Lett., 2019, 30, 1307-1309.

6 P.B. Zhang, M. Zhu, M.H. Geng-Spyropoulos, M. Shardell, M. Gonzalez-Freire, V. Gudnason, G. Eiriksdottir, D. Schaumberg, J. E. Van Eyk, L. Ferrucci and R. D. Semba, Proteomics, 2017, 17.