A Ratiometric Biosensor for Metallothionein Based on Dual Heterogeneous Electrochemiluminescent Responses from TiO₂ Mesocrystals formed interface

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Chemicals and Materials. Metallothionein was purchased from Abcam (Cambridge, UK). Nafion 117 solution (5% w/v mixture of low molecular weight alcohols) was purchased from Sigma-Aldrich. Phosphate buffer solution (PBS) was prepared by mixing stock solution of 0.1 M NaH2PO4 and 0.1 M Na2HPO4 and adjusting the pH. Other reagents were of analytical regent grade. All solutions were prepared with deionized water by a Milli-Q water purification system (Millipore, Milford, MA, USA).

Apparatus. ECL intensity versus potential was detected by using a system made in our laboratory, consisting of a BPCL Ultra-Weak Chemiluminescence Analyzer controlled by a personal computer with BPCL program (Institute of Biophysics, Chinese Academy of Sciences) in conjunction with a CHI 760 electrochemical analyzer (Shanghai Chenghua Instrument Co., China). The electrochemical analyzer was used for controlling waveforms and potentials. A conventional three-electrode system was used for the electrolytic system, including a bare GCE or modified GC electrode as the working electrode, a platinum wire as the counter electrode and Ag/AgCl electrode (sat. KCl) as the reference electrode. A commercial 5 ml cylindroid glass cell was used as ECL cell, and

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it was placed directly in the front of the photomultiplier tube. The crystal structure of the products were characterized by X-ray powder diffraction (XRD) (XRD-6000, Shimadzu Instrument, Japan) with Co-K α radiation (λ = 1.78897 Å), and the data were changed to Cu-K α data. Scanning electron microscopy (SEM, Hitachi S-4800) was used to observe the morphologies and sizes. Transmission electron microscopy (TEM, FEI F20 S-TWIN), high-resolution TEM (HRTEM) and the selected area electron diffraction (SAED) analysis were performed on a JEOL-2100 transmission electron microscope.

Synthesis of TiO₂ Mesocrystals. The synthesis of TiO₂ mesocrystals was composed of two steps. At first, titanate nanowires were prepared by hydrothermal growth in basic aqueous solution and acid-washed, which was similar to the previous report. 1.5 g of anatase TiO₂ was dissolved in a 75 mL of 15 M KOH solution. After the solution was stirred for 10 min, it was transferred to a 100 mL Teflon-lined autoclave. After that, the reactor was held at 170 °C for 72 h in an oven and was cooled naturally to room temperature. Then, the precipitate was obtained. After that the resulting precipitate was washed several times with 0.1 M H₂SO₄ solution to the pH of resulting precipitate reached 2. The prepared titanate nanowires were collected by centrifugation and air-dried at 70 °C for 12 h. The second step, titanate nanowires (60 mg) were dispersed into 20 mL of 1 M H₂SO₄ solution. The solution was under stirring at 70 °C for 7 days. Then, the final products were collected by centrifugation and washed absolutely with distilled water and air-dried at 70 °C for 12 h for further use.

Syntheses of CdTe QDs. A series of aqueous colloidal CdTe solution were prepared using the reaction between Cd^{2+} and NaHTe solution following the method described elsewhere. $CdCl_2$ (0.182 g , 1mmol) and MPA (0.27 mL) were dissolved in 75 mL of deionized water, and adjusted the pH to 10 with 1M NaOH. The solution was placed in a three-necked flask, fitted and was deaerated with N₂ bubbling for 30min. Then, freshly prepared NaHTe solution (0.5mmol) was quickly injected into the mixture under vigorous stirring, followed by refluxing

under nitrogen atmosphere. The resulting mixture solution was heated to 96°C and refluxed 2 hours. Aliquots of the reaction solution were taken out at regular intervals for subsequent experiments.

Construction of dual-responses ECL immunsensor. The bare glassy carbon electrode (GCE, 3 mm in diameter) was polished with 0.3, 0.05 μ m alumina particles on chamois leather in sequence, then washed sequentially with doubly distilled water in an ultrasonic bath and dried in air before use. For preparation of TiO₂-Nafion modified electrode, 0.05% Nafion solution was added into the 3 mg/ml TiO₂ solution, followed by ultrasonication for 5min. With a microinjector, 4 μ L of TiO₂-Nafion solution was deposited on the fresh prepared GCE surface, and kept in an over at 60°C for 25min. Then the prepared electrode was immersed in 0.1 mM Ru(bpy)₃²⁺ solution for 30 min. MT was immobilized on the sensing interface by immersing the modified electrode in MT solution (0.8 μ M) for 40 min at room temperature, and then incubated into CdTe quantum dots solution (0.1wt.%) for about 30min at room. The ECL sensing strategy for the detection of MT was showed in schemel. The prepared modified electrode could be stored in refrigerator at 4 °C when not in use.

The mechanism of the dual-responses ECL biosensor

The possible mechanisms of two ECL emissions were investigated in the work. The anodic ECL of $Ru(bpy)_3^{2+}$ was assigned to the reaction between the low concentration of OH⁻ in PBS and high concentration of $Ru(bpy)_3^{2+}$ accumulated in Nafion film:

$$\operatorname{Ru}(\operatorname{bpy})_{3}^{2^{+}} - e^{-} \longrightarrow \operatorname{Ru}(\operatorname{bpy})_{3}^{3^{+}}$$
(1)

$$Ru(bpy)_{3^{3+}} + OH^{-} \longrightarrow Ru(bpy)_{3^{2+*}} + 1/2H_2O + 1/4O_2$$
 (2)

$$\operatorname{Ru}(\operatorname{bpy})_{3}^{2+*} \longrightarrow \operatorname{Ru}(\operatorname{bpy})_{3}^{2+} + \operatorname{h} v \tag{3}$$

 $Ru(bpy)_{3}^{3+}$ was electrogenerated from $Ru(bpy)_{3}^{2+}$ and immobilized in the Nafion layer due to its strong electrostatic interaction with the Nafion. Then $Ru(bpy)_{3}^{3+}$ reacted with OH⁻ to give rise to the persistent ECL. The cathodic

ECL was produced upon concomitant reduction of CdTe QDs and $S_2O_8^{2-}$. CdTe QDs were reduced to CdTe • ⁻ by the electron injection upon the negative potential, while the coreactant $S_2O_8^{2-}$ was reduced to the strong oxidant $SO_4 \cdot ^-$. After that, the as-produced CdTe • ⁻ and $SO_4 \cdot ^-$ radicals interacted at once to form high eigenstate CdTe* species that produced light emission. The related reactions of ECL emission were described as following:

$$CdTe + e^{-} \longrightarrow CdTe \cdot -$$
(4)

$$S_2O_8^{2-} + e^- \longrightarrow SO_4 \cdot \cdot + SO_4^{2-}$$
 (5)

$$CdTe \cdot + SO_4 \cdot - \longrightarrow CdTe^* + SO_4^{2-}$$
(6)

$$CdTe^* \longrightarrow CdTe + hv \qquad (7)$$



Figure S-1 (A) SEM image of TiO_2 MC, the inset is TEM image, (B) HRTEM images of the TiO_2 MC, (C) XRD patterns of TiO_2 MC, (D) the BJH pore size distribution of the TiO_2 MC (a), N₂ adorption-desorption isotherms of the TiO_2 MC (b).

Characterization of TiO₂ mesocrystals. TiO₂ mesocrystals was successfully synthesized in the supporting information. Then, various characteristic techniques were proved insight into the morphologies and structures of TiO₂ MC. Figure S-1A showed an image of TiO₂ MC, indicating the large scale nanoparticles with a size ranged from 25 nm to 50 nm as well as rough surface, which constructed the octahedral structure. The typical TEM image of a single nanoparticle was shown in the inset of Figure S-1A. The image displayed that the TiO₂ nanoparticles possessed a truncated-octaherdral-shape and was composed of tiny nanoparticle subunits. In addition, the HRTEM image shown in Figure S-1B depicted that the obvious lattice fringes at 0.36 and 0.47 nm were assigned to (101) and (002) planes of the anatase structure, respectively. The related XRD pattern was shown in Figure S1C, exhibiting that the diffraction peaks were well defined to tetragonal anatase TiO₂ indexed to JCPDS 65-5714. The

acid aqueous solution was origin of the product with a different phase. On the basis of the broadened diffraction peaks, the cell parameters were calculated, and the lattice constants of a=0.378 nm and c=0.943 nm were obtained. To further explore the porous nature of TiO₂ MC, we used Brunauer-Emmett-Teller and Barrett-Joyner-Halenda (BET/BJH) methods to determine specific surface area and porosity. The N₂ adsorption-desorption isotherm (Figure S-1D (a)) shows characteristics of the mesocrystal. The sharp uptake at relative pressures P/P0<0.05 proved that micropores were present in the mesocrystal. Moreover, an H₁ hysteresis loop at P/P₀>0.7 indicated the presence of mesopores. The BET surface area and the total pore volume are 211.07 m² g⁻¹ and 0.43 cm⁻³ g⁻¹, respectively, which is much larger than other TiO₂ mesocrystals reported in the previous literatures. More interestingly, curve b in the Figure S-1D revealed that a single peak in BJH pore distribution was about 19 nm, indicating that very uniform mesopores can be obtained with the simple method. Thus, TiO₂ mesocrystals possessed uniform mesopores successfully absorbed more Ru(bpy)₃²⁺ to extend outer Helmholtz plane of the sensing interface, and make the pH in the composite film higher, resulting in Ru(bpy)₃²⁺ possessing strong ECL emission in moderate condition.



Figure S-2 (A) the time of $Ru(bpy)_{3}^{2+}$ immobilization on Nafion / GCE(a), Nafion-TiO₂ MC / GCE (b), (B) Nyquist diagrams of electrochemical impedance spectra of various electrodes. Electrolyte: 5mM [Fe(CN)₆]^{3-/4-} and 0.1 M KCl. Nafion/GCE (a), Nafion-TiO₂ MC/GCE (b), Nafion-TiO₂ MC-MT /GCE (c), Nafion-TiO₂ MC-MT -CdTe/GCE (d). (C) Effect of the concentration of TiO₂ MC; (D) Effect of the concentration of Nafion;

Electrochemical impedance was carried out to further characterise various modified electrodes. In electrochemical impedance measurement, the semi-circle diameter of impedance equals the electron transfer resistance. Figure S-2B showed the Nyquist diagrams of Nafion/GCE(a) and Nafion-TiO₂ MC/GCE(b), Nafion-TiO₂ MC-MT/GCE(c) and Nafion-TiO₂ MC-MT-CdTe/GCE(d) in 5 mM Fe(CN)₆^{3-/4-} containing 0.1M KCI. It could be seen that obviously well-defined semi-circles at higher frequencies were obtained at all these electrodes. The impedance values of Nafion/GCE was biggest, suggesting that Nafion film blocks the charge migration between electrode surface and Fe(CN)₆^{3-/4-}. When TiO₂ MC and Nafion composite film was introduced onto GCE, the electron transfer resistance was effectively reduced, indicating that TiO₂ MC as semiconductor possessed good conductivity, and the porosity of the mesocrystals could not only make the modified film to be more porous, but also accelerated the charge migration and improve the surface wettability between electrode and electrolyte similar to the performance above. However, when MT was immobilized on Nafion-TiO₂/GCE, the semi-circle dramatically enlarged, suggesting that the largely molecular MT was insulative. The performance proved that the MT was successfully assembled on the electrode through covalent reaction between -SH of the MT and TiO₂ mesocrystals. Then CdTe QDs were immobilized on Nafion-TiO₂-MT/GCE, the impedance value decreased obviously, resulting from semiconductor CdTe QDs possessed the good conductivity and the CdTe QDs were embedded in the MT structure, which might form an electron tunneling to promote the electron transfer.



Figure S-3 the assembled time of MT on the ECL responses of $Ru(bpy)_3^{2+}$ (A) and CdTe QDs (B) at Nafion-TiO₂ MC-MT-CdTe/Ru(bpy)₃²⁺/GCE; the assembled time of CdTe QDs on the ECL responses of $Ru(bpy)_3^{2+}$ (C) and CdTe QDs (D) at Nafion-TiO₂ MC-MT-CdTe / $Ru(bpy)_3^{2+}$ / GCE; The effect of the pH value of PBS on the ECL responses of $Ru(bpy)_3^{2+}$ (E) and CdTe QDs (F) on Nafion-TiO₂ MC-MT-CdTe/Ru(bpy)₃²⁺/GCE.

Condition Optimization. To optimize the experimental conditions for MT detection, the effects of the amount of TiO₂ MC and Nafion were investigated. As it depicted in Figure S-2C, the ECL intensity enhanced obviously with the increasing amount of TiO₂ MC on the Nafion-TiO₂ MC/Ru(bpy)₃^{2+/}GCE. When the amount was beyond the 3 mg/mL, the response decreased. It was suggested the more mesocrystals would result in the mesocrystals aggregated together and lower the electric conductivity and blocked the electron transfer. Figure S-2D illustrated that the ECL response increased with increasing concentration of Nafion from 0.03% to 0.075%, then decreased with further increased of Nafion concentration. It was well known that the most popular method for immobilization was based on an ion exchanging technology, which immobilizes the ECL material, normally Ru(bpy)₃²⁺, by using Nafion cationic exchange polymer. So the more Nafion, the more Ru(bpy)₃²⁺ was immobilized in the film. Then the ECL response increased. But Nafion film blocked the electron transfer, it was so much amount of Nafion that ECL response decreased. When both of them reach a balance, the biggest signal was obtained. Thus, 3 mg/mL TiO₂ MC and 0.075% Nafion (w/v) were chose to modify the electrode and used as the modified layer components in following experiments. In addition, the assembled time of MT and CdTe QDs was the important experiment factor, as displayed in Figure S-4A and Figure S-4B. The ECL emission of Ru(bpy)₂²⁺ decreased with the increasing assembled time of MT, but the ECL intensity of CdTe QDs enhanced with the increasing the assembled time. Both of their responses retained stable, when the time exceeded 45 min. Figure S-4C and Figure-S4D showed the effect of assembled time of QDs, which was similar to the effect of assembled time of MT on the ECL response. When the time got to 30 min, both of the responses of Ru(bpy)32+ and QDs became stable. Therefore, we chose 45 min and 30 min to assemble MT and CdTe QDs, respectively. The effect of the pH value of PBS on the ECL responses of Ru(bpy);²⁺ and CdTe QDs on Nafion-TiO₂ MC-MT-CdTe/Ru(bpy)₃²⁺/GCE were also investigated in the pH range from 6.5 to 9.0 (Figure S-4E). As can be seen in Figure S-4E, when the when the pH value of buffer solution increased from 6.5 to 8.0, the ECL intensity of Ru(bpy)₃²⁺ at modified electrode increased gradually. When the pH was further beyond 8.0, the ECL intensity conversely decreased. However, the ECL intensity of $Ru(bpy)_{3}^{2+}$ at modified electrode enhanced with pH increasing from 6.5 to 7.4, as shown in Figure S-4F. When the pH was beyond 7.4, the response decreased. Therefore, taking into account further application under physiological conditions, pH 7.4 PBS was used as the detection solution.

Table S-1 Comparable figures of determining MT

Method	Linear ranges (ng/mL)	Detection limit (ng/mL)	Reference
QCM	20-250	20	2
ELISA	3-100	3	20
HPLC	70-100	70	20
ECL	5-10000	1.67	This work

QCM Quartz crystal microbalance; ELISA enzyme-linked immunosorbent assay; HPLC high-performance liquid chromatography.

Sample	Found	Added	Total found	Recovery
	(ng/mL)	(ng /mL)	(ng/mL)	(%)
1	10.1669	10.0000	20.1518	99.8
2	10.1635	10.0000	20.1740	100.1