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Homogeneous electrochemiluminescence immunoassay of tris (2, 3–dibromopropyl) isocyanurate using luminol luminescence and Ti/TiO_2 NTs electrode

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Supplementary Information

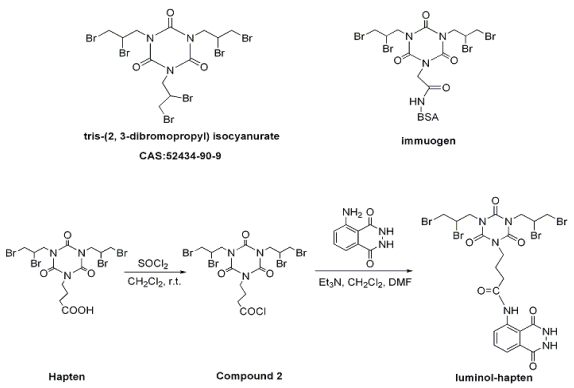
Experiment section

Chemicals and Reagents. Titanium foil (99.8 % purity, 0.127 mm thick) was purchased from Aldrich (Milwaukee, WI). Anti–TBC antibody was derived from immune rabbit preparation in this laboratory using the immunogen shown in Scheme 1. Hapten and immunogen were synthesized in house. $NaHSO_4$, NaF, Tris(2,3–dibromopropyl) isocyanurate (TBC), luminol, pentabromodiphenyl ether (PBDE), naphthalene (Nap), hexabromobenzene (HBB), anthracene (Ant), fluorine (Flu), perfluorooctane sulphonate (PFOS) and hexachlorobenzene (HCB), as well as other reagents of analytical reagent grade were all obtained from commercial sources and used as received. Phosphate buffer solution (PBS) was prepared with NaH_2PO_4 and Na_2HPO_4. Twice distilled water was used throughout the experiment.

Apparatus. A field emission scanning electron microscope (FESEM, Hitachi S–4800) was used for studying the TiO_2 NTs morphologies. The infrared spectrum was obtained on a FT–IR spectrophotometer (Thermo Nicolet 5700). UV–vis absorption spectrum was obtained with a UV/vis/NIR spectrophotometer (Varian CARY 300 Conc).

Synthesis of luminol–hapten conjugate. The high stability and the high symmetry of TBC make it difficult to be derivated. A hapten shown in Scheme 1 was therefore used, and labeled with luminol according to the routine shown in Scheme **S1**. To the solution (8 mL) of hapten (165 mg, 0.268 mmol) solution in dichloromethane was added dropwise thionyl chloride (0.1 mL, 99.5 %, 1.18 mmol). The reaction mixture was stirred overnight at room temperature and then concentrated in vacuo to produce a colorless oil (Compound **2**, 150 mg, yield: 88 %) which was used without further purification.

The resulted Compound **2** was dissolved in dichloromethane (3 mL) in which the solution in DMF (3 mL) of 0.277 mmol aminophthalhydrazide and 0.2 mL triethylamine were added. Then the reaction mixture was stirred for 6 h at room temperature and concentrated in vacuo. The residue was purified by a silica gel column eluted with dichloromethane-methanol (40:1, v/v) to give the luminol–hapten, a white solid (65 mg, 35 % yield).



Scheme S1. Chemical structures of hapten, immunogen, TBC as well as the synthetic procedure of luminol–hapten.

Preparation of polyclonal anti–TBC antibody. The immunization procedure followed the protocol reported previously.[1,2] The antibodies were obtained by immunizing New Zealand male rabbits with the immunogen shown in Scheme **S1**. The final blood was collected about 4 months following the first immunization. Blood was collected in test tubes, and blood samples were left to coagulate for 1 h at 37 °C and 1 h at 4 °C, followed by centrifugation at 10000 rpm for 10 min. Part of the

polyclonal antiserum was purified following the previous methods.[3] The titer and inhibition of the resulted antibody detected by ELISA were 1:6400 and 10 μg/mL, respectively.

Homogeneous ECLIA measurement. TiO_2 nanotubes were prepared by anodizing titanium foils at a constant potential of 20 V in an electrolyte containing 0.1 M NaF and 0.5 M $NaHSO_4$ for 2 h in a two–electrode configuration with a platinum cathode.[4] Before anodization, the titanium foil was pretreated by sonicating in 3% HF solution for several minutes, then washed in water. After anodization the TiO_2 NTs film was immediately washed with water and next dried in air.

The ECL intensity was measured with a MPI–E electrochemical workstation (China) using a three–electrode system: the TiO_2 NTs film with a geometrical area of 1.5 ± 0.01 cm^2 as the working electrode, a Pt wire counter electrode, and a saturated Ag/AgCl reference electrode. The voltage of the photomultiplier tube (PMT) was set at 700 V.

Luminol–hapten dissolved in 0.1 M NaOH containing 5% (v/v) acetonitrile was used as the stock solution. The work solution of luminol–hapten was prepared to 1.9 μM by diluting the stock solution with 0.01 M PBS (pH 7.4). The homogeneous ECLIA measurement was conducted in a 6 mL cell containing 4 mL luminol-hapten work solution. The ECL intensity of the luminol–hapten work solution was firstly measured as (I_a), which was used as the internal standard. TBC at different concentrations and anti–TBC antibody at a final concentration of 1.25 mg/L were added into the work solution. After incubating the solution at 25 °C for 120 min, the ECL intensity was measured as (I). The ECL intensity of a solution without addition of TBC was defined as the blank response (I_0). The relative increase in ECL intensity, $(I–I_0)/I_0$, resulted from the addition of TBC was used to quantify the TBC concentration. In order to minimize the instrumental diversity, all the measured ECL values were normalized with the internal standard, (I_a), i.e. I/I_a or I_0/I_a was used instead of I or I_0.

Characterization section

Luminol–hapten dissolved in 0.1 M NaOH containing 5% (v/v) acetonitrile was used as the stock solution. The work solution of luminol–hapten was prepared to 1.9 μM by diluting the stock solution with 0.01 M PBS (pH 7.4). The synthesized luminol–hapten was well characterized by UV–vis, FT–IR, 1H NMR and MS spectra. The UV–vis spectrum (Fig. **S1**A) shows that luminol exhibits two UV–vis absorption peaks at 300 and 350 nm (curve a), and luminol–hapten has a peak at 350 nm (curve b), while TBC exhibits no obvious UV–vis absorption peaks (curve c). The modification results in the disappearing of the peak at 300 nm. The FT–IR spectra shown in Fig. **S1**B displays the characteristic bands of luminol–hapten between 3350–3184 cm^{-1} ($\nu_{N–H}$), 2923–2852 cm^{-1} ($\nu_{C–H}$), 1697 cm^{-1} ($\nu_{C=O}$, ν_{COOR}), 1630–1458 cm^{-1} ($\nu_{C=C}$, in aromatic ring) and 1310–763 cm^{-1} ($\nu_{C–N}$, $\nu_{C–O}$, $\nu_{C–Br}$). 1H NMR (d6-DMSO, 400 MHz): 1.92~1.98 (m, 2H), 2.84 (t, J = 7.20 Hz, 2H), 3.70~3.76 (m, 2H), 3.91~3.96 (m, 2H), 4.02~4.09 (m, 2H), 4.20~4.29 (m, 4H), 4.52~4.59 (m, 2H), 6.77 (d, J = 7.60 Hz, 1H), 6.95 (d, J = 7.60 Hz, 1H), 7.40 (bs, 1H), 7.51 (t, J = 8.00 Hz, 1H), 12.08 (s, 1H); MS(EI) m/z (%): 774.0 (M+, 1%).

SEM images in Fig. **S1**C show that the as-anodized TiO_2 NTs are highly oriented grown on the Ti film with a size of 320 nm in length (inset of Fig. **S1**C) and an average pore size of 90 nm. These results are consistent with our previous studies.[5–7] Such a nanotuberale structure is with high surface area, benefiting the adsorption of reactants on the surface and charges transfer.

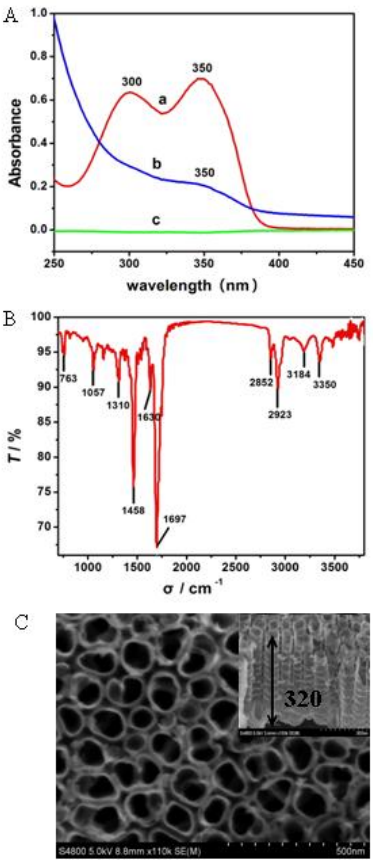


Fig. S1. (A) UV–vis spectra of luminol (a), luminol–hapten (b) and TBC (c); (B) FT–IR spectra of luminol–hapten; (C) SEM image of the TiO_2 NTs.

Optimization of experimental conditions

To achieve a high ECL signal, experimental conditions including buffer ionic strength, antibody concentration, pH, as well as incubation time and temperature were optimized.

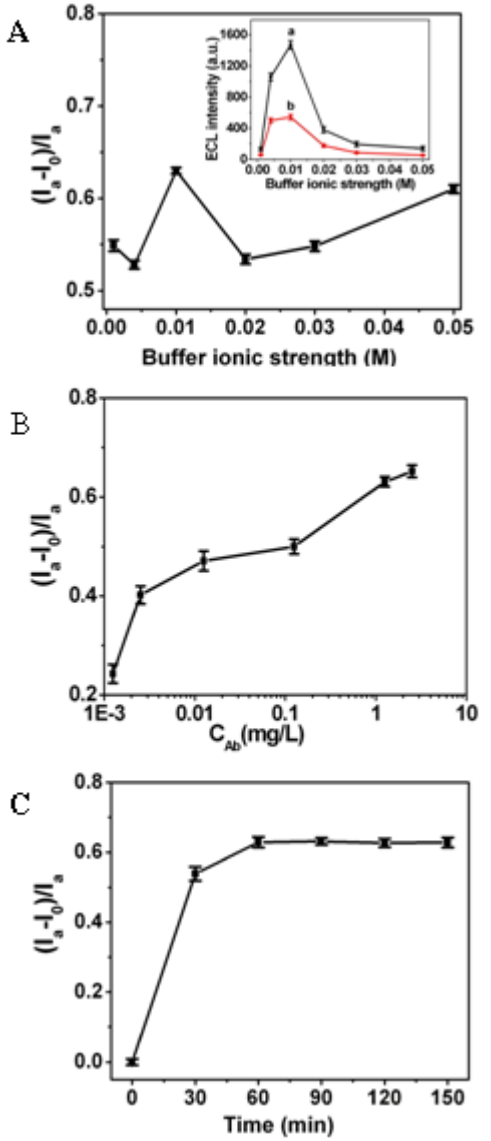


Fig. S2. Effect of ionic strength (A), the antibody concentration (B), and the incubation time (C) on the relative ECL responses in 0.01 M PBS (pH 7.4) solution containing 1.9 μM luminol–hapten. The incubation time for (A) and (B) is 120 min, and the antibody concentration for (A) and (C) is 1.25 mg/L. Inset of Fig. 5 (A) shows the ECL responses in the absence (curve a) and presence (curve b) of anti–TBC antibody.

The ionic strength is a crucial parameter that affects the ECL intensity. The effect of ionic strength was investigated with PBS (pH 7.4) solution at various concentrations ranging from 0.001 to 0.05 M, containing 1.9 μM luminol–hapten, and 1.25 mg/L anti–TBC antibody or not. After incubation for 120 min, the ECL intensity was measured. Fig. **S2**A shows the relationship between the ionic strength and the relative ECL changes. Generally, the optimal ionic strength is the one at which the relative ECL change, i.e $(I_a–I_0)/I_a$ is the maximum. One can see that the $(I_a–I_0)/I_a$ value is not too much dependent on the ion strength since both the (I_a) (in the absence of antibody) and (I_0) values (in the presence of antibody) have the similar change trends with the ion strength. However, another factor is needed to be considered, i.e. the absolute change in the ECL intensity $(I_a–I_0)$. A relatively large $(I_a–I_0)$ value would be good for decreasing the detection errors because a small $(I_a–I_0)$ value would easily cause accidental errors. As shown in the inset of Fig. **S2**A, the ECL intensity of luminol–hapten no matter in the presence of antibody or not increases gradually with increasing ionic strength from 0.001 to 0.01 M, and then decreases with further increasing ionic strength over 0.01 M. The low ECL intensity in high concentration electrolyte solution is because that the electrolyte of high concentration can inhibit the ECL reaction of luminol.[8–10] The maximum $(I_a–I_0)$ value is obtained at 0.01M PBS solution. Such an ionic strength can ensure a high accuracy and sensitivity. Therefore, a 0.01 M PBS solution was used in the following analysis.

A key in competitive immunoassay is the antibody concentration, which has to be in limiting amount to saturate the antigen. Fig. **S2**B shows the antibody concentration –dependent ECL changes relative to the ECL of luminol. The ECL was measured after incubating 0.01 M PBS (pH 7.4) solution containing 1.9 μM luminol–hapten and antibody for 120 min. Basically, the optimal antibody concentration is the one at which a less change in antibody concentration can cause a larger change in the ECL intensity, i.e. the point at which there is the maximum slope in Fig. **S2**B. One can see that the maximum slope is achieved at the antibody concentration of 0.0025 mg/L. However another factor is needed to be considered, i.e. the antibody–caused absolute change in the ECL intensity, the value of $(I_a–I_0)$, because a small $(I_a–I_0)$ value would easily cause accidental errors. One can see that both a relatively large $(I_a–I_0)$ value and a large slope are obtained at the antibody concentration of 1.25 mg/L. Such a concentration can ensure a high accuracy and sensitivity, as well as a wide detection range. Thus, the antibody concentration of 1.25 mg/L was used to perform the competition.

In homogeneous ECLIA, pH, electrolyte concentration, temperature, and the incubation time for antigen–antibody interaction greatly influence the sensitivity. Considering the practical applications of the proposed system in environmental analysis, all experiments were carried out in pH 7.4 solution at RT (25 ± 1.0 °C). Following that, the effect of incubation time of

the immunoreaction (from 0 to 150 min) on the responses was investigated. A 0.01 M PBS (pH 7.4) solution containing 1.9 μM luminol–hapten and 1.25 mg/L anti–TBC antibody was incubated at 25 °C for different time. As shown in Fig. **S2**C, the response increases with increasing the incubation time upto 60 min, and then trended to level off after 60 min. The longer incubation time could not improve the response. In order to ensure that the immunoreaction can be performed completely, 90 min of incubation time was used for the detection of TBC in this study.

The recovery rates

The recovery rates were investigated by analyzing water samples spiked with TBC in the concentrations of 3.4 nM, 17 nM and 85 nM, respectively. Recovery rate is defined as the ratio of the added amount detected to the actually added amount, i.e $R=(m_a–m_b)/m$, where m_a/m_b the detected value after/before addition of the target, and m the added amount of target. Table S1 lists the recovery rates.

Table 1. Recovery study for TBC using homogeneous ECLIA with various water samples

Sampe	Added (nM)	Detectd (nM)	Recovey (%)	RSD (%)
Tap water	0	ND[a]		
	3.4	3.1	91.2	6.33
	17.0	15.4	90.6	4.92
	85.0	81.9	96.4	3.86
River water	0	ND		
	3.4	3.2	94.1	5.53
	17.0	15.8	92.9	6.12
	85.0	80.3	94.5	3.24
Waste water	0	5.3[b]		
	3.4	8.9	105.9	7.36
	17.0	22.6	101.8	5.69
	85.0	87.1	96.2	4.71

[a]ND: not detected;

[b]The TBC content in the waster water is calculated to be 0.53 nM based on the detected value.

Real water sample determination

The practicability of the method was evaluated by determinating the waste water collected from the outlet of a TBC manufacturing plant along Liuyang River (Liuyang, Hunan province, China). As shown in Fig. **S3**, the enriched waster water sample causes a significant change in the ECL intensity, while the enriched river water sample causes insignificant change in the ECL intensity. The TBC content in the outlet of a TBC manufacturing plant along Liuyang River is calculated to be 0.53±0.03 nM as shown Table 1.

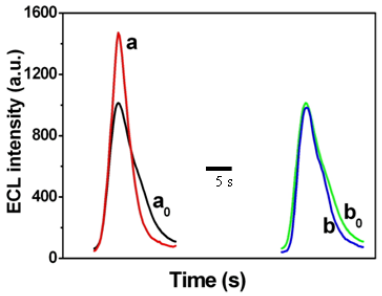


Fig. S3. Water samples determination: (a_0) absence and (a) presence of enriched waste water samples and (b_0) absence and (b) presence of enriched Liuyang river water samples in air–saturated 0.01 M PBS (pH 7.4) containing 1.9 μM luminol–hapten and 1.25 mg/L anti–TBC antibody.

Notes and references

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