Electronic Supplementary Information (ESI)

In-Electrode *vs*. On-Electrode: Ultrasensitive Faradaycage-Type Electrochemiluminescence Immunoassay

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Experimental Section

1. Reagents

Bovine serum albumin (BSA), neurotensin (NT) and polyclonal neurotensin antibody (anti-NT), N-(4-aminobutyl)-N-ethylisoluminol (ABEI), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), hydrogen peroxide (H_2O_2, 30 wt%) and ammonium hydroxide (NH_4OH, 25 wt%) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Track-etched membranes were purchased from Shenghechengxin Membrane Technol. Co. (Beijing, China). All other reagents were of analytical grade or above and used without further purification. Carbonate buffer solution (CBS, pH 9.74) containing 0.2 mol/L Na_2CO_3, 0.2 mol/L $NaHCO_3$ and 1 mmol/L H_2O_2, was used as the electrolyte solution for ECL measurement. Ultrapure water obtained from Millipore water purification system (≥18 MΩ cm, Milli-Q, Millipore, Billerica, MA, USA) was used

throughout the experiment.

2. Apparatus

The ECL measurements were detected with a custom-made setup consisting of a BPCL Ultra-Weak Chemiluminescence Analyzer (Institute of Biophysics, Chinese Academy of Sciences, Beijing, China) and a CHI 660A electrochemical analyser (Shanghai Chenhua Instrument Co., Shanghai, China), which was controlled by a computer. A three-electrode system including a bare or modified magnetic glass carbon working electrode (3 mm diameter, Gaossunion, Wuhan, China), platinum wire auxiliary electrode and Ag/AgCl (3 mol/L KCl) reference electrode, was employed. Electrochemical impedance spectroscopy (EIS) analysis was carried out with a CHI 660E electrochemistry workstation (Chenhua Instrument Company, Shanghai, China) using the same three-electrode system as that of the ECL detection. Experimental conditions of EIS were: magnetic glass carbon electrode in 0.05 mol/L PBS (5 mmol/L $[Fe(CN)_6]^{3-/4-}$, pH 7.4); and frequency range from 0.01 to 100,000 Hz with a signal amplitude of 5 mV. Scanning electron microscope (SEM, Hitachi SU-70, Tokyo, Japan) and atomic force microscope (AFM, Multimode 8 Bioscope system, Brucker) were used to characterize the morphology of graphene oxide (GO), g-C_3N_4, magnetic GO (nanoFe_3O_4@GO) and multi-functionalized GO.[1]

3. Synthesis of nanoFe_3O_4@GO

In a typical experiment, uniform size GO was prepared by acid oxidation of graphite powder according to the modified method reported by Chen *et al*. (Figure S3).[2] NanoFe_3O_4@GO was prepared as reported by a previous report.[3] In brief, 40 mg

of GO was dispersed in 40 mL of water and ultrasonicated for 30 min in a round-bottom flask. After that, 50 mL of iron source solution containing 800 mg of $FeCl_3 \cdot 6H_2O$ and 300 mg of $FeCl_2 \cdot 4H_2O$ was added, and the mixture solution was vigorously stirred for 1 min at room temperature under a stream of nitrogen. The solution was heated to 85 °C and ammonium hydroxide (25 wt%) was added dropwise increasing the pH to 10. Then, the mixture solution was rapidly stirred for 45 min while its color changed from brown to black. Subsequently, the resulted black nanoFe_3O_4@GO was washed thoroughly with water to neutral pH using magnet and dried under vacuum at room temperature. SEM image of nanoFe_3O_4@GO composite is shown in Figure S2A. It was observed that Fe_3O_4 nanoparticles were nearly spherical and homogeneously distributed over the GO sheets.

4. Preparation of multi-functionalized graphene oxide

Capture unit (Ab_1-nanoFe_3O_4@GO): First, 10 mg of nanoFe_3O_4@GO was dissolved in 2 mL of water by continuous ultrasonication to obtain a homogeneous suspension. Then, 200 µL of mixture solution containing 100 mg/mL EDC and 10 mg/mL NHS was added into 200 µL of the above solution. After adjusting the pH to 5.0, the solution obtained was shaken for 1 h at ambient temperature to form a stable active ester layer on the surface of GO, and the resulted black precipitate was thoroughly washed using a magnet with water for 3 times. Subsequently, 50 µL of 1:5000 Ab_1 was added and the pH was adjusted to 9.0, followed by shaking for 4 h. Later, 100 µL of 2 wt% BSA was added to block non-specific binding sites. Finally, Ab_1-nanoFe_3O_4@GO composite was obtained after washing and reconstructing in 200

μL of water. SEM image of capture unit composites was shown in Figure S2B. It was observed that the composite film became much rougher and the structure of film was completely changed.

Detector unit ((Ab_2&ABEI)@GO): An amount of 200 μL of 1 mg/mL GO was mixed with 400 μL of a solution containing 100 mg/mL EDC and 10 mg/mL NHS. To this mixture, 0.5 μL of 1 mol/L HCl was added and incubated for 1 h under constant shaking. The unreacted reagents were purified twice by centrifugation at 8000 rpm for 15 min with water. Then, 30 μL of 28 mg/mL ABEI and 50 μL of 1: 5000 Ab_2 were added, shaken for another 4 h, and then 100 μL of 2 wt% BSA was employed to block non-specific adsorption. The final brown production was separated by centrifugation at 8000 rpm for 15 min and resuspended in 50 μL of water, and referred as (Ab_2&ABEI)@GO.

5. Preparation of the Electrochemiluminescence (ECL) immunosensor

The bare magnetic glass carbon electrode was polished successively with 1.0, 0.3 and 0.05 μm alumina slurry, washed ultrasonically in anhydrous ethanol and water in succession, and allowed to dry using N_2. Then, 5 μL of Ab_1-nanoFe_3O_4@GO composite solution was dropped on the electrode. Within a few minutes, the composite was attracted on the surface of the magnetic glass carbon electrode.

6. ECL detection

The ECL immunosensor was incubated in 5 μL of NT samples for 1 h at 37 °C, carefully washed with water to remove unbound NT and then incubated in 5 μL of (Ab_2&ABEI)@GO at 37 °C for 1 h to form the final Faradaycage-type composite.

When a chronoamperometry (30 s pulse period, 0.25 s pulse width, 0 V initial potential, and 1 V pulse potential) was applied to the working electrode in the electrolyte solution (0.05 mol/L CBS at pH 9.74 containing 1mmol/L H_2O_2), an ECL signal was generated and recorded with the voltage of the photomultiplier at 700V.

7. Preparation of g-C_3N_4

First, the g-C_3N_4 bulk material was prepared following the previously reported literature.[4] Briefly, melamine was placed in an alumina crucible with a cover and then heated at 600 °C for 4 h. The obtained yellow product was the bulk g-C_3N_4 powder. Then, g-C_3N_4 nanosheets were synthesized as follows: to 1 g of this bulk g-C_3N_4 powder, 100 mL of 5 mol/L HNO_3 was added, refluxed for 24 h at 125 °C and allowed to cool at room temperature. The white product was centrifuged at 12000 rpm, washed with ultrapure water to near-neutral pH, and redispersed in water. Then the resultant suspension solution was sonicated for 4 h and then centrifuged at 8000 rpm for 30 min to remove the residual unexfoliated g-C_3N_4 and large-area nanosheets before use. Finally, the product was dried in a vacuum oven for 12 h at 35 °C to obtain the carboxylated g-C_3N_4 nanosheets. Pure g-C_3N_4 has very poor luminescence intensity due to its poor conductivity.[5]

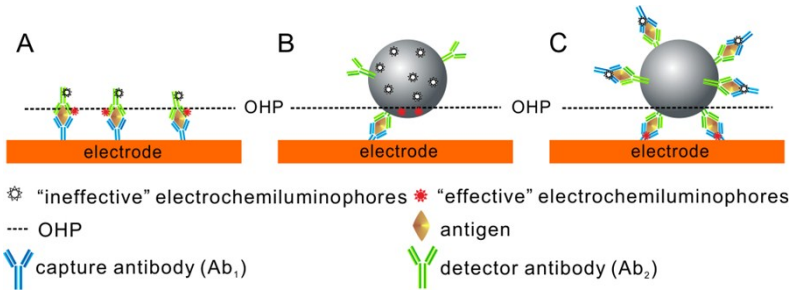

Figure S1 Factors restricting the sensitivity of the conventional "on-electrode" sandwich-type ECLIA.

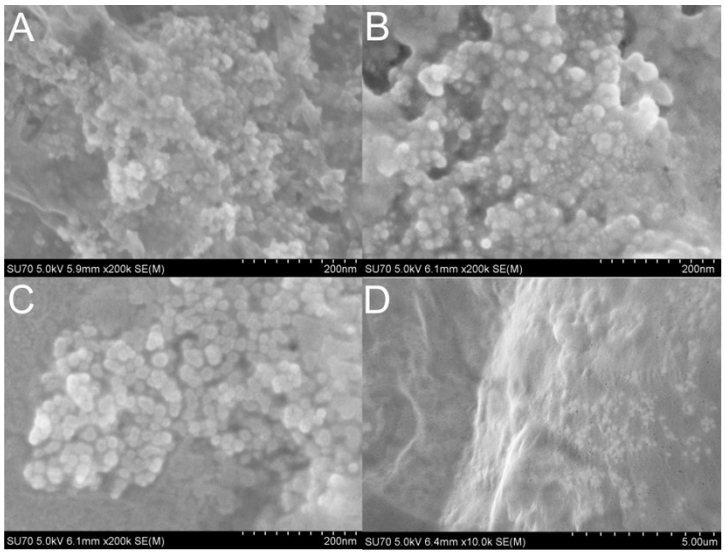

Figure S2. Representative SEM images of (A) nanoFe_3O_4@GO, (B) capture unit, (C) NT-capture unit, and (D) detector unit-NT-capture unit.

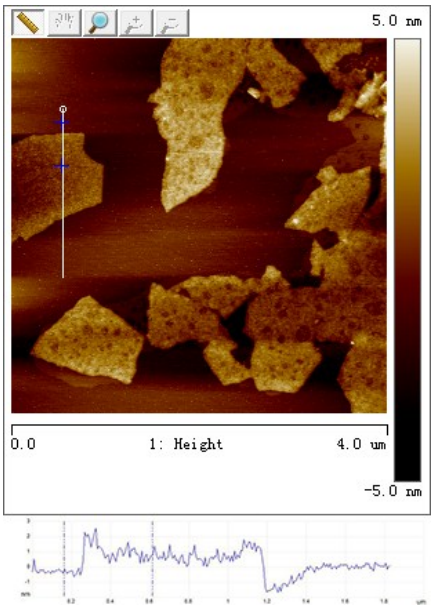

Figure S3. Atomic force microscopy (AFM) image of GO. Line chart in the bottom of the AFM image shows associated height profiles.

The proposed "in-electrode" Faradaycage-type ECLIA method was achieved using GO, which is a single atom thick and two dimensional carbon nanomaterial. GO nanosheet in particular plays a significant role in our strategy through extending the OHP of the electrode effectively. The surface morphology of GO was characterized by AFM (Figure S3) and a neat surface made up of unfolded GO nanosheets is clearly observed. The associated height profiles display that the average height of the homogeneous GO nanosheets is approximately 1 nm.

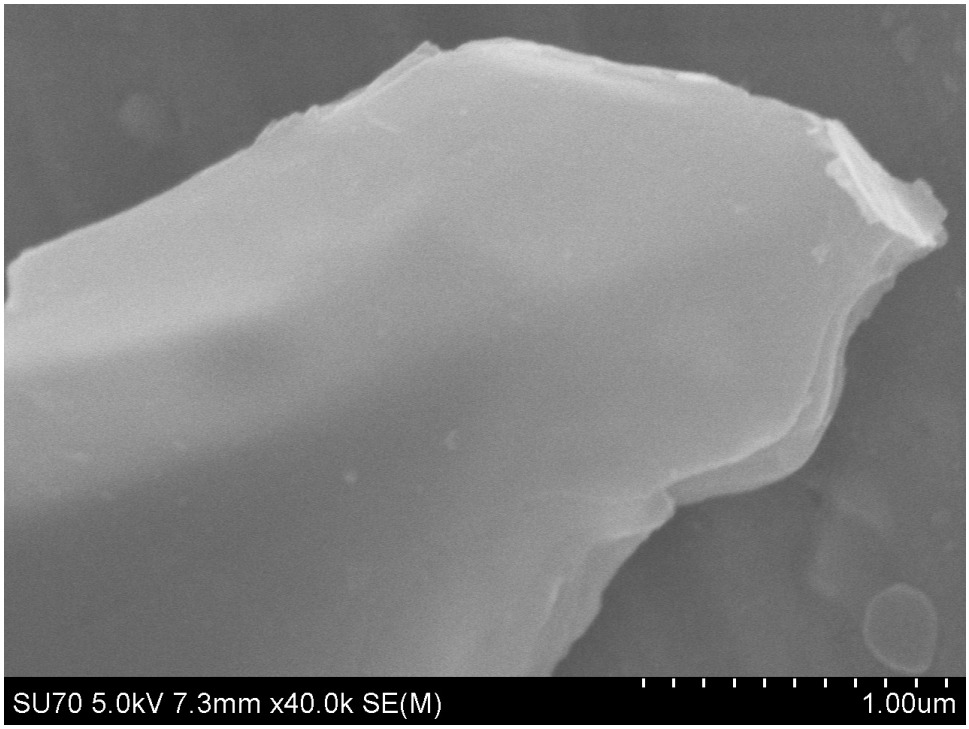

Figure S4. SEM image of g-C_3N_4.

Morphology of the prepared carboxylated g-C_3N_4 nanosheets exhibits good flake-like shape (Figure S4), the size of which was similar to that of GO prepared.

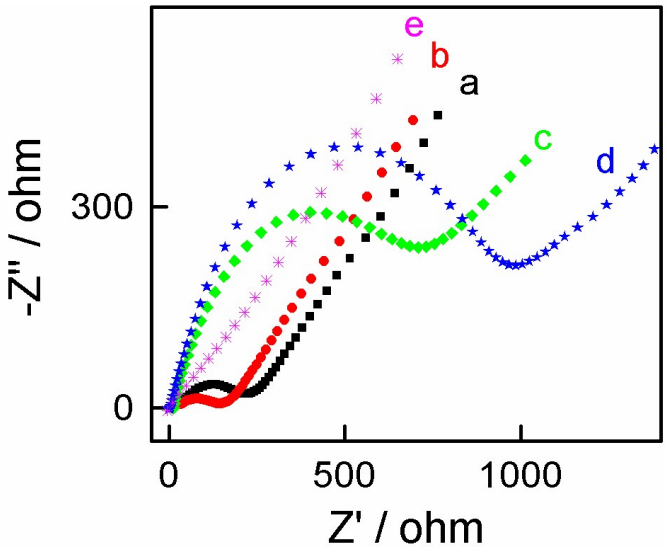

Figure S5. Electrochemical impedance spectra of the different modified electrodes: (a) magnetic glassy carbon electrode (MGCE), (b) nanoFe_3O_4@GO/MGCE, (c) capture unit/MGCE, (d) NT–capture unit/MGCE and (e) detector unit–NT–capture unit/MGCE. The concentration of NT is 5 pg/mL.

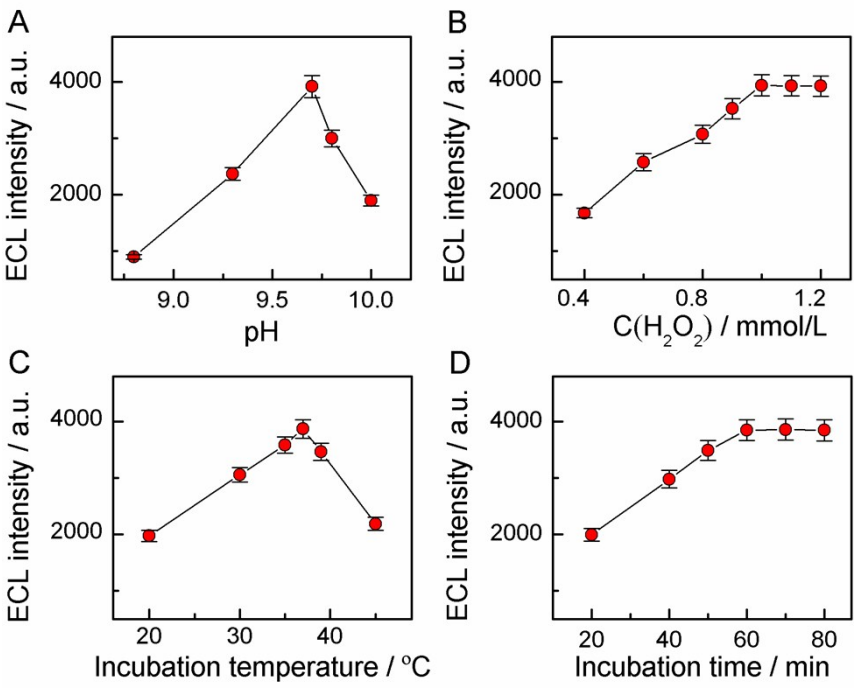

Figure S6. Effect of (A) pH; (B) concentration of H_2O_2; (C) incubation temperature; and (D) incubation time on the ECL intensity of the Faradaycage-type immunosensor. Assays were conducted using 5 fg/mL NT solution.

To apply multi-functionalized graphene oxide in the ECL immunoassay, several experimental parameters, including the pH of the detection solution, concentration of H_2O_2 in electrolyte solution, incubation temperature and the incubation time of antigen were optimized with the concentration of NT at 5 fg/mL.

The ECL performance of ABEI greatly depends on the solution. The effect of pH in the range of 8.8 − 10.0 (CBS, 0.05 mol/L) was examined. As shown in Figure S6A, the optimal pH was 9.74 as the ECL intensity reached its maximum value. Therefore, pH 9.74 was used in the following experiments.

The ECL reaction of ABEI in alkaline solution was markedly improved by the addition of H_2O_2 over the range of 0.4 − 1.2 mmol/L (Figure S6B). The reason is that

ABEI deprotonates in alkaline solution to form an anion that can undergo electrochemical oxidation. The intermediate species obtained undergoes further electro-oxidation in the presence of H_2O_2 to produce an excited state, which produces the ECL emission finally. Thus, the ECL intensity increased with the increase of the H_2O_2 concentration and reached the maximum at 1 mmol/L.

The effect of incubation temperature was investigated in the range of 20 − 45 °C. The ECL intensity increased and reached the maximum value 37 °C, after which the ECL intensity decreased rapidly (Figure S6C). Therefore, 37 °C was selected as the optimal incubation temperature.

The effect of incubation time of antigen was also investigated in the range of 20 − 80 min as shown in Figure S6D. When the incubation time was longer than 60 min, the ECL intensity did not increase with the increasing incubation time because the reaction was almost completed.

Therefore, the optimal experimental conditions of this assay were as follows: 0.05 mol/L CBS at pH 9.74 containing 1 mmol/L H_2O_2, 37 °C incubation temperature and 60 min incubation time of antigen.

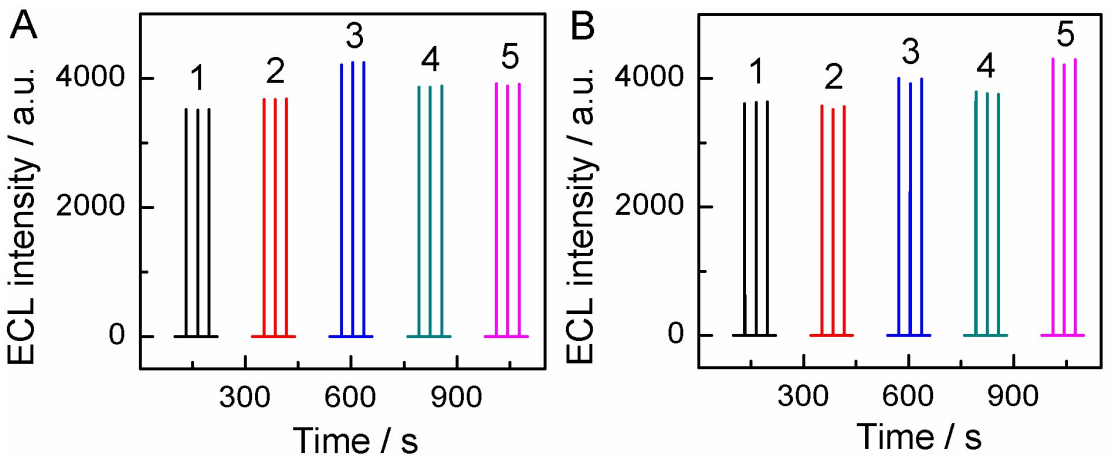

Figure S7 The reproducibility of the proposed immunosensor. (A) The intra-assay precision evaluated from the response to 5 fg/mL NT at five different immunosensors fabricated in the same batch. (B) The inter-assay precision evaluated from the response to 5 fg/mL NT with five proposed immunosensors prepared with different batches.

Table S1 Comparison of main methods reported in the literature for the determination of NT.

Analytical methods	Detection range	LOD	References
HPLC[a]	5 ~ 5000 ng/mL	2 ng/mL	6
TOFMS[b]	117.18 ~ 1791 μg/mL	117.18 μg/mL	7
ELISA[c]	1673 ~ 16730 ng/mL	1673 ng/mL	8
ECIA[d]	5 ~ 1670 pg/mL	3 pg/mL	9
ECIA[d]	20 ~ 10^5 pg/mL	6 pg/mL	10
ECLIA[e]	0.001 ~ 100 pg/mL	0.001 pg/mL	11
ECLIA[e]	$5 \times 10^{-3} \sim 5 \times 10^4$ fg/mL	0.002 fg/mL	This study

[a] HPLC (High Performance Liquid Chromatography)

[b] TOFMS (Time-of-Flight Mass Spectrometry)

[c] ELISA (Enzyme-Linked Immunosorbent Assay)

[d] ECIA (Electrochemical Immunosensors)

[e] ECLIA (Electrochemiluminescence Immunoassay)

Table S2 Measurement of NT spiked in 10% blood serum or urine (v/v) by the proposed electrochemiluminescence immunoassay

Samples	Added (fg/mL)	Found[a] (fg/mL)	RSD[a] (%)	Recovery[a] (%)
Serum 1	500	479 ± 24.4	5.1	95.8
Serum 2	5	5.54 ± 0.57	10.3	110.8
Serum 3	0.05	0.058 ± 0.007	12.1	116.0
Urine 1	500	543 ± 39.6	7.3	108.6
Urine 2	5	4.72 ± 0.32	6.8	94.4
Urine 3	0.05	0.056 ± 0.006	10.7	112.0

[a] the average value of three parallel determinations.

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