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Terbium Doped Graphitic Carbon Nitride Endows Highly Sensitive Ratiometric Fluorescence Assay of Alkaline Phosphatase Activity

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Experimental Details

Chemicals and materials

Thiourea, magnesium sulfate ($MgSO_4$), calcium chloride ($CaCl_2$), zinc chloride ($ZnCl_2$), ammonium chloride (NH_4Cl), sodium chloride (NaCl), potassium chloride (KCl), ferric trichloride ($FeCl_3$), ferrous sulfate ($FeSO_4$), ascorbic acid (AA), glucose, phenylalanine (Phe), lysine (Lys), threonine (Thr), isoleucine (Ile), leucine (Leu), tryptophan (Trp), methionine (Met), valine (Val) and nitric acid (HNO_3) were obtained from Sinopharm Chemical Reagent Co. (Shenyang, China). *p*-nitrophenol (PNP), *p*-nitrophenyl phosphate (PNPP), terbium(III) chloride hexahydrate ($TbCl_3 \cdot 6H_2O$), glutathione (GSH), Adenosine 5'-triphosphate (ATP) and transferrin (TRF) were purchased from Aladdin Industrial Corporation (Shanghai, China). Alkaline phosphatase (ALP), human serum albumin (HSA), acetyl cholinesterase (AChE), lysozyme (LZM) and pepsin (Pep) were obtained from Sigma-Aldrich (St Louis, MO, USA). Dicarboxyl-terminated PEG (HOOC-PEG-COOH, M.W. 2000) was provided by Ponsure Biotech (Shanghai, China). Trypsin (Try) was purchased from Labgic Technology Co., Ltd. (Hefei, China). Sodium orthovanadate (Na_3VO_4) was obtained from Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China). All the reagents were used as received without further purification. Deionized water (DI) of 18 MΩ cm^{-1} was used throughout.

Instrumentations

The morphology and size of the prepared nanosheets were observed on a JEM-2100 HR transmission electron microscope (TEM, JEOL Ltd., Japan) and a SU8010 field

emission scanning electron microscope (SEM, Hitachi, Japan). X-ray diffraction (XRD) patterns were obtained by using an Empyrean X-ray diffractometer (Panalytical, Netherlands) with Cu-Ka irradiation in the range of 2θ from 5 to 90. FT-IR spectra were recorded by using a VERTEX70 FT-IR spectrophotometer (Bruker, Germany) from 400 to 4000 cm^{-1}. The surface charge properties of g-C_3N_4:Tb nanosheets in aqueous media were measured by a ZS90 Nano Zetasizer (Malvern, UK). UV-vis absorption spectra were recorded on a UH5300 spectrophotometer (Hitachi, Japan) with a 1.0 cm quartz cell. The photoluminescence (PL) measurements were performed using an F-7000 fluorescence spectrophotometer (Hitachi, Japan) providing a 1.0 cm quartz cell. The excitation and emission slits were both set as 10 nm with a scan speed of 2400 nm min^{-1}. The quantum yields of g-C_3N_4:Tb and its derivative were obtained by using a Quantarus-QY absolute photoluminescence quantum yield measurement system (Hamamatsu Photonics, Japan). The number of HepG2 cells was measured by Countess™ II FL automated cell counter (Thermo Fisher Scientific, USA).

Preparation of g-C_3N_4:Tb nanosheets

The g-C_3N_4:Tb nanoprobe is prepared by following the procedure in the previous work with minor modifications.[1] 0.761 g (10 mmol) of grinded thiourea was put into a 250 mL beaker and dissolved in 10 mL of water, followed by the addition of 75 mg of $TbCl_3 \cdot 6H_2O$ and 10 μL of nitric acid (16 M). The reaction mixture was immediately irradiated under microwave for 3 min in a domestic microwave oven (G80F23CN3L-Q6(P0), Galanz, China) at output power of 800 W. The cream white powder, g-

C_3N_4:Tb, was obtained and washed thoroughly with DI water to remove residual acid on the surface of materials. The g-C_3N_4:Tb nanosheets were obtained by exfoliating the bulk g-C_3N_4:Tb by ultrasound for 48 h. The suspension was then centrifuged at 3000 rpm for 15 min to remove the un-exfoliated g-C_3N_4:Tb with large size.

The modification of g-C_3N_4:Tb nanosheets with HOOC-PEG-COOH

10 mg of the as-prepared g-C_3N_4:Tb were re-dispersed in 10 mL of DI water under ultrasonication followed by mixing with 0.25 mL of HOOC-PEG-COOH solution (20 mg mL^{-1}) at magnetic stirring for 12 h. HOOC-PEG-COOH was adsorbed onto the surface of g-C_3N_4:Tb nanosheets via electrostatic interaction. The product g-C_3N_4:Tb-PEG nanosheets were collected and purified by washing with DI water for 3 times followed by centrifugation at 5000 rpm.

Ratiometric fluorescence assay of ALP with g-C_3N_4:Tb-PEG nanosheets as the probe

The ratiometric fluorescence detection of ALP was assessed according to the following operations. ALP with different activities (0 to 30 U L^{-1}) was added into 10 mM Tris-HCl buffer solution (pH 9.5) containing 100 μg mL^{-1} g-C_3N_4:Tb-PEG nanosheets, 1.5 mM PNPP and 2 mM $MgSO_4$. After incubation at 37°C for 120 min, the fluorescence spectra were recorded within 320-560 nm at the excitation wavelength of 290 nm. Fluorescence intensity for the intrinsic emission of native g-C_3N_4 (Em_1) at 355-400 nm is denoted as F_1, and that of g-C_3N_4:Tb-PEG nanosheets (Em_2) at 546 nm is given as F_2. The ratio of F_2 and F_1 (F_2/F_1) is defined as the output signal to correlate with the activities of ALP. All the quantitative detections were

in triplicate. Scheme S1 illustrated the mechanisms of the ratiometric fluorescence strategy with g-C_3N_4:Tb-PEG nanosheets as probe for the assay of ALP activity based on inner filter effect.

Optimization of experimental conditions

In order to receive a high sensitivity for the subsequent detection, the four variables (pH, Mg^{2+} concentration, PNPP amount and temperature) were investigated. 5 U L^{-1} ALP was added into 10 mM Tris-HCl buffer solution containing PNPP (0-3 mM), $MgSO_4$ (0-10 mM) and 100 μg mL^{-1} g-C_3N_4:Tb-PEG nanosheets. After being incubated for 120 min, the fluorescence spectra were recorded in 320-560 nm at the excitation wavelength of 290 nm.

Selectivity of g-C_3N_4:Tb nanosheets for ALP detection

To investigate the selectivity of the assay probe over other substances, certain levels of metal ions, biomolecules, amino acids and enzymes were introduced into the assay system. The details of the potential interferents include: 150 mM Mg^{2+}, Ca^{2+}, NH_4^+, Zn^{2+}, Na^+, K^+, Cl^- and SO_4^{2-}, 0.015 mM of Fe^{2+} and Fe^{3+}, 0.1 mM of GSH, AA, glucose, ATP and amino acids (Phe, Lys, Thr, Ile, Leu, Trp, Met and Val), 10 mg mL^{-1} of HSA and TRF. The activities of Try, Pep, LZM and AChE were 1 mg mL^{-1}. The investigation method was based on the ALP activity assay procedure as described above.

ALP inhibitor investigation

To study the application of our established ratiometric fluorescence approach for ALP inhibitor evaluation, Na_3VO_4 solution with different concentrations (0 to 600 μM)

were added into 100 μg mL^{-1} g-C_3N_4:Tb-PEG nanosheets solution containing 5 U L^{-1} ALP, 10 mM Tris-HCl buffer solution (pH 9.5) and 2 mM $MgSO_4$. The mixture was incubated at 37°C for 30 min and then 1.5 mM PNPP was introduced. The above prepared sample was incubated at 37°C for another 120 min and then its fluorescence intensity was measured from 320 nm to 560 nm at the excitation wavelength of 290 nm. The inhibition efficiency (%) was expressed as:

$$\text{Inhibition efficiency (\%)} = \frac{\left(\frac{F_2}{F_1}\right)_1 - \left(\frac{F_2}{F_1}\right)_0}{\left(\frac{F_2}{F_1}\right)_B - \left(\frac{F_2}{F_1}\right)_0}$$

$(F_2/F_1)_B$, $(F_2/F_1)_0$, and $(F_2/F_1)_1$ denote the fluorescence ratio for the mixture of g-C_3N_4:Tb-PEG nanosheets and PNPP, (B) in the absence of either ALP or the inhibitor, (0) in the presence of ALP while in the absence of the inhibitor, and (1) in the presence of both ALP and the inhibitor.

ALP assay in serum

The serum samples herein included human serum and livestock serum. The human blood samples were collected from three healthy adult volunteers at the School Hospital of Northeastern University. The blood samples were then centrifuged at 3500 rpm for 10 min to obtain human serum samples before ALP assay. The livestock serum sample (rabbit) was obtained from a healthy rabbit. The study protocols were reviewed and approved by Animal and Medical Ethics Committee of Northeastern University, China. The livestock serum sample (fetal bovine) was purchased from Clarkbio. Co. (Shanghai, China). The same procedure as described above is followed to perform ALP activity assay. Meanwhile, for the purpose of comparison, the serum

samples were also analyzed by the commonly used ALP kit with colorimetric detection.

In vitro biocompatibility assay of g-C_3N_4:Tb-PEG nanosheets

The biocompatibility was studied by an MTT assay. First, the HepG2 cells, cultured in DMEM (HyClone, China) supplemented with 10% fetal bovine serum (FBS), 1% penicillin and 1% streptomycin, were firstly seeded in 96-well plates (1.0×10^4 cells per well) and incubated for 24 h in a humidified incubator (5% CO_2, 37 ℃). Then, the cells were incubated with different concentrations of g-C_3N_4:Tb-PEG (10, 20, 50, 100, 200 and 400 μg mL^{-1}). After incubation for 20 h, an MTT solution (5.0 mg mL^{-1}) was added in each well and the cells were cultured at 37℃ for another 4 h. When the purple precipitate was clearly visible, 150 μL DMSO was added to dissolve the generated formazan crystals and was swirled gently. Thereafter, absorbance of each well was measured at 490 nm by a microplate reader (BIO-TEK, USA).

Detection of ALP activity in HepG2 cell lysates

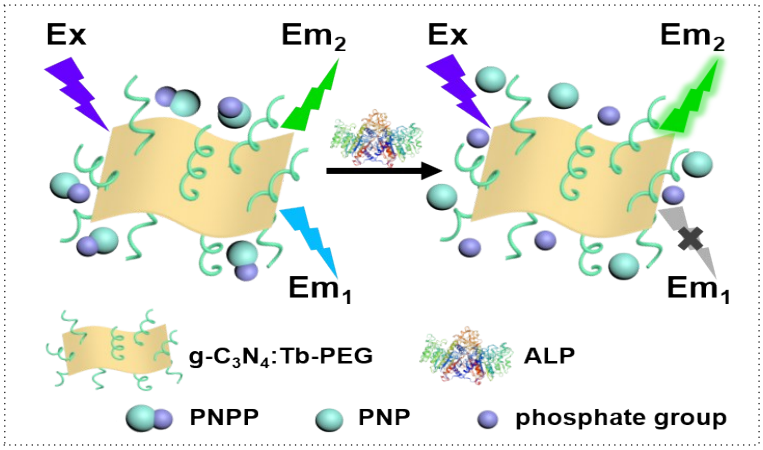
Liver cancer cells (HepG2) were obtained from Chinese Academy of Sciences Cell Bank (Shanghai, China). To implement ALP detection in cancer cells, the cell populations were collected and lysed by ultrasonic cell crusher at 400 W. The lysis is performed for 3 s followed by a stop of 10 s, and this process is alternately conducted for 30 times. The cell lysis is followed by centrifugation at 10000 rpm for 10 min to obtain 1.0 mL of supernatant. Thereafter, the cell lysate was appropriately diluted and added into the detection system. Then the reaction mixture was incubated at 37°C for

2h. The same procedure as that for the analysis of ALP in aqueous

medium was afterwards applied for cellular ALP quantification.

Statistical analysis

The obtained analytical data are displayed as mean ± standard deviation (SD). The data collected from the real samples are analyzed by Student's t-test for the comparison of the two methods used, i.e., the present ratiometric fluorescence detection and the commercial ALP kit with colorimetric detection. GraphPad Prism 6.01 is used to perform the above statistical analyses.



Scheme S1. The illustration for the ratiometric fluorescence strategy with g-C_3N_4:Tb-PEG nanosheets as the probe for the detection of ALP activity based on inner filter effect.

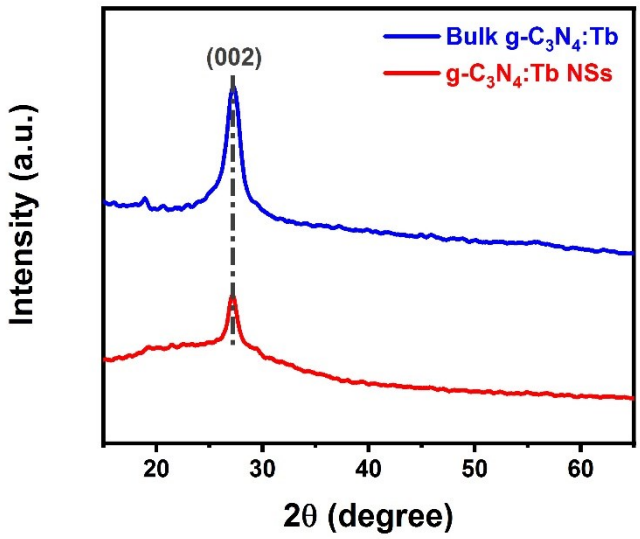


Figure S1. XRD patterns of the bulk g-C_3N_4:Tb and the g-C_3N_4:Tb NSs after ultrasonic treatment.

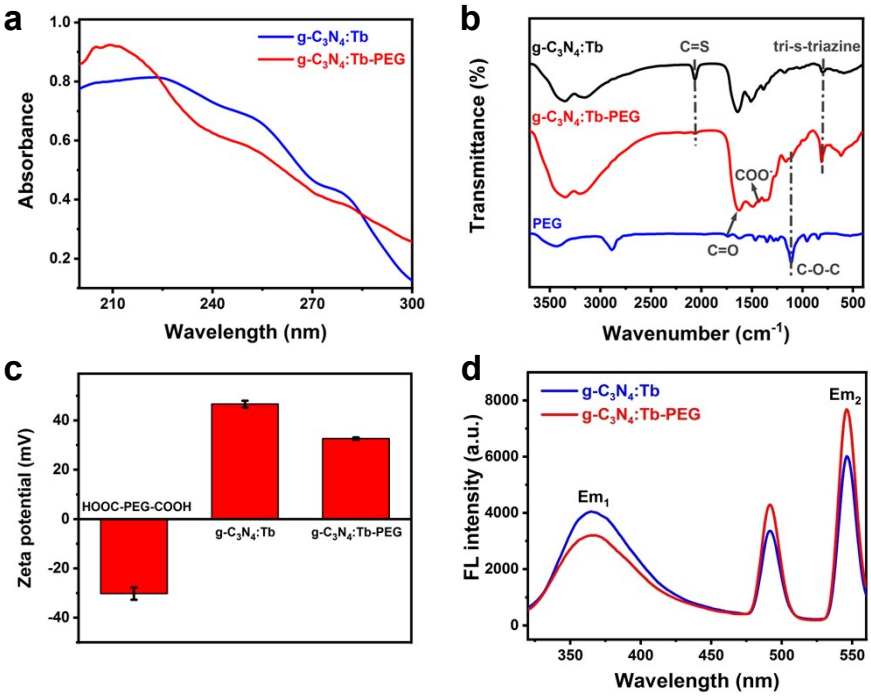


Figure S2. (a) UV-vis absorption spectra of g-C_3N_4:Tb and g-C_3N_4:Tb-PEG (25 μg mL^{-1}). (b) FT-IR spectra of HOOC-PEG-COOH, g-C_3N_4:Tb and g-C_3N_4:Tb-PEG. (c) Zeta potential of HOOC-PEG-COOH, g-C_3N_4:Tb and g-C_3N_4:Tb-PEG aqueous solutions (at pH 7). (d) Fluorescence spectra of g-C_3N_4:Tb and g-C_3N_4:Tb-PEG (100 μg mL^{-1}, λ_{ex} 290 nm).

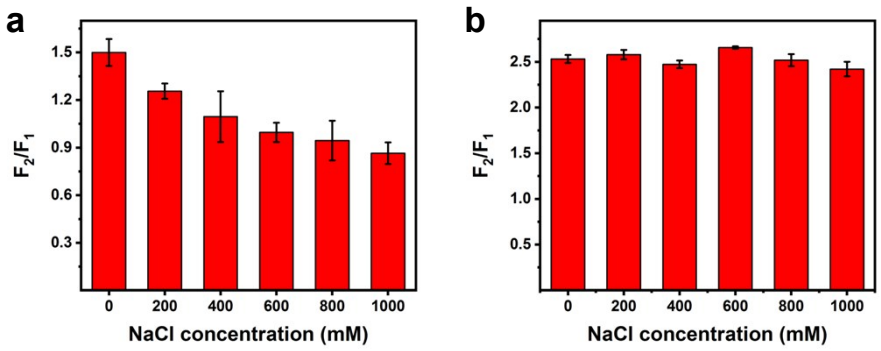


Figure S3. The variation of ratiometric fluorescence responses of 100 μg mL^{-1} (a) g-C_3N_4:Tb nanosheets and (b) g-C_3N_4:Tb-PEG nanosheets with various concentrations of NaCl in the range of 0-1000 mM.

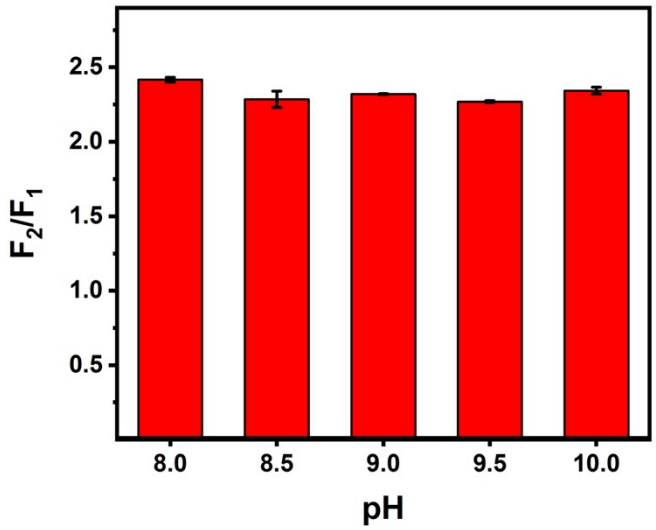


Figure S4. The ratiometric fluorescence response of g-C_3N_4:Tb-PEG NSs (100 μg mL^{-1}) under different pHs.

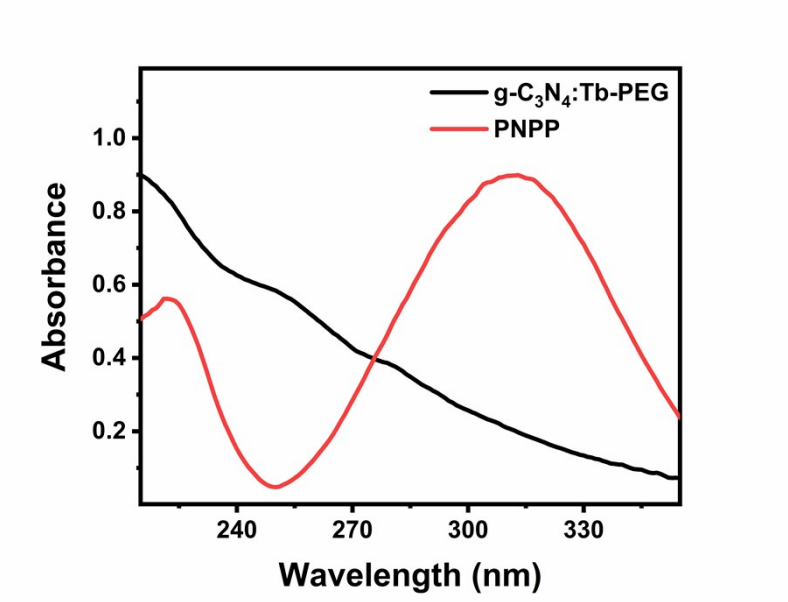


Figure S5. The absorption spectra of g-C_3N_4:Tb-PEG nanosheets and PNPP.

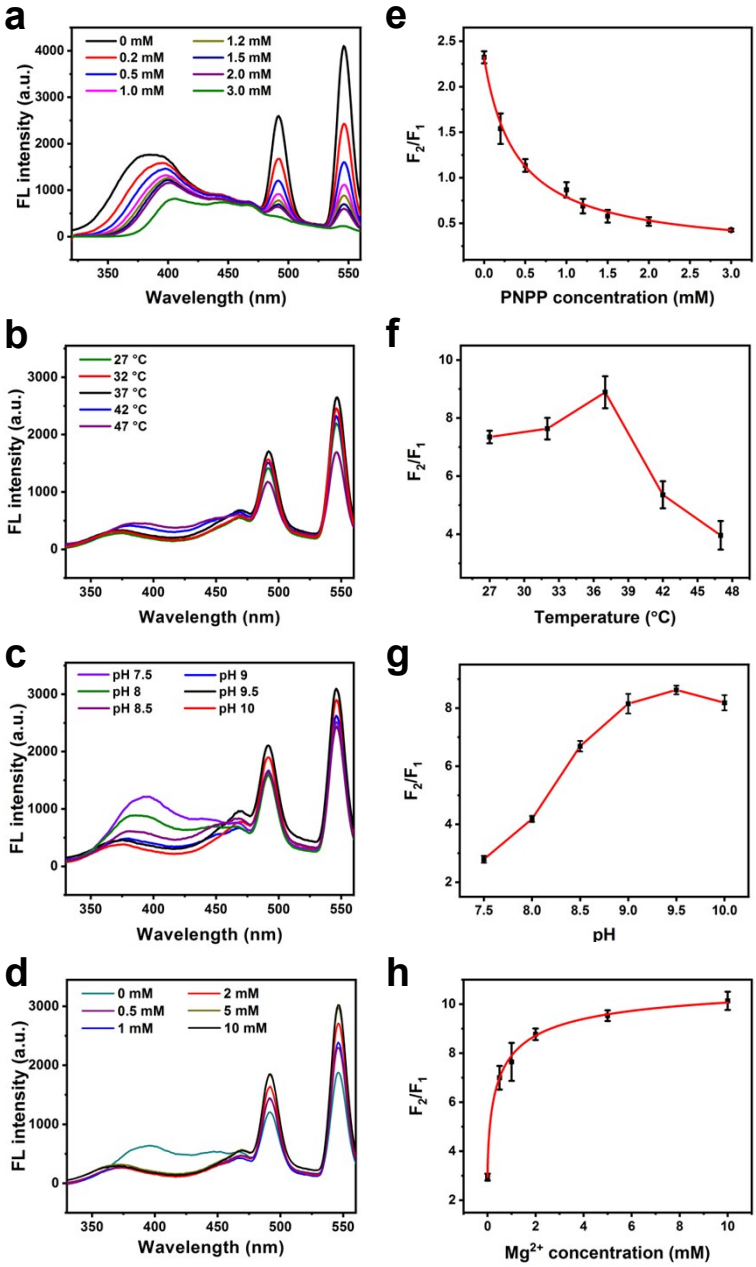


Figure S6. The left column: the effect of (a) PNPP concentration, (b) temperature, (c) pH, (d) Mg^{2+} concentration to the fluorescence spectra of g-C_3N_4:Tb-PEG NSs (100 μg mL^{-1}). The right column: the effect of (e) PNPP concentration, (f) temperature, (g) pH, (h) Mg^{2+} concentration to the ratiometric fluorescence response of the probing system based on IFE.

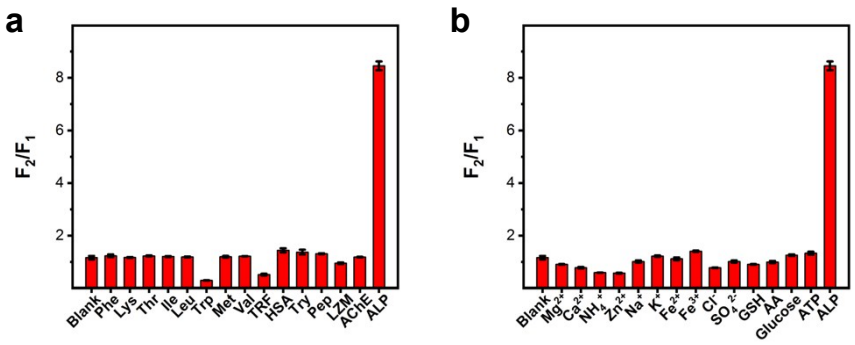


Figure S7. Ratiometric fluorescence response (F_2/F_1) of the g-C_3N_4:Tb-PEG NSs-based assay system toward various coexisting species, including (a) amino acids (0.1 mM Phe, Lys, Thr, Ile, Leu, Trp, Met, Val), proteins (10 mg mL^{-1}, HSA, TRF), enzymes (1 mg mL^{-1}, Try, Pep, LZM, AChE), (b) cationic and anionic species (150 mM Mg^{2+}, Ca^{2+}, NH_4^+, Zn^{2+}, Na^+, K^+, Cl^-, SO_4^{2-}; 15 mM Fe^{2+}, Fe^{3+}), small molecules (0.1 mM GSH, AA, Glucose, ATP) and ALP (5 U L^{-1}).

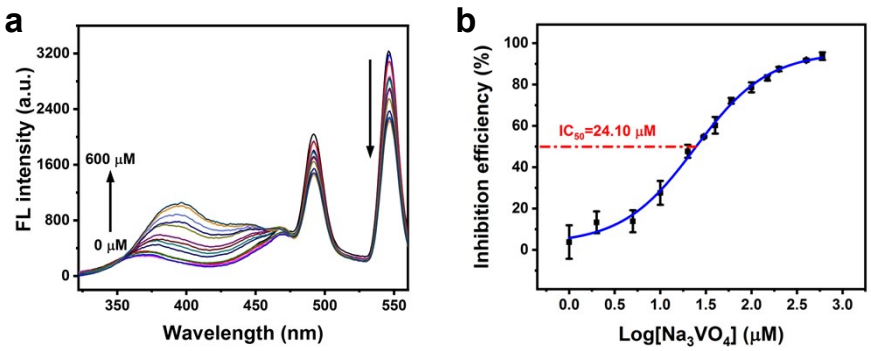


Figure S8. (a) Fluorescence responses of g-C_3N_4:Tb-PEG nanosheets in the presence of various concentration of Na_3VO_4 (0-600 μM). (b) Inhibition efficiency as a function of the logarithmic concentration of Na_3VO_4.

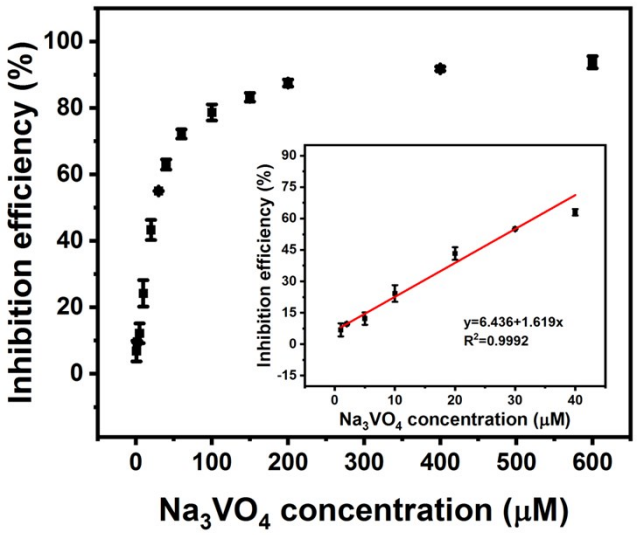


Figure S9. Fluorescence responses of g-C_3N_4:Tb-PEG NSs in the presence of various concentration of Na_3VO_4 (0-600 μM). Inset: the linear relationship between the inhibition efficiency and Na_3VO_4 concentration in the range of 1-40 μM.

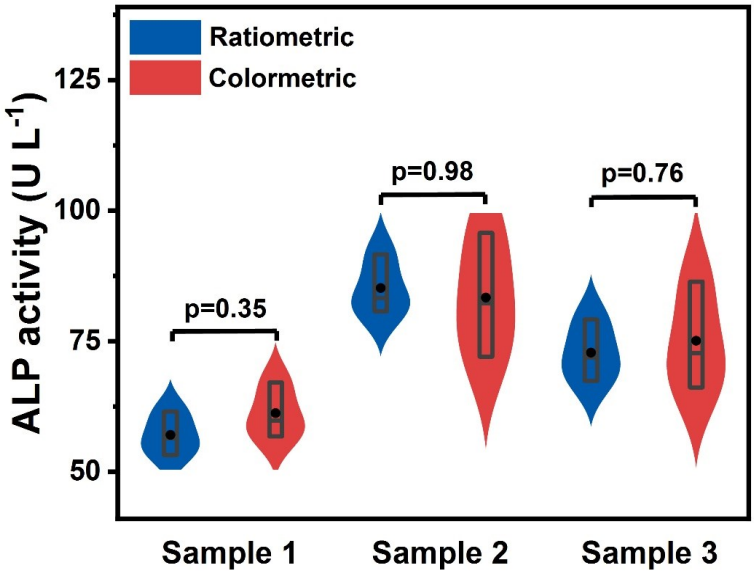


Figure S10. Violin plot of the difference in ALP activity of different samples detected by ratiometric fluorescence and colorimetry (Students' t-test by GraphPad Prism 6, P>0.05, no significant difference).

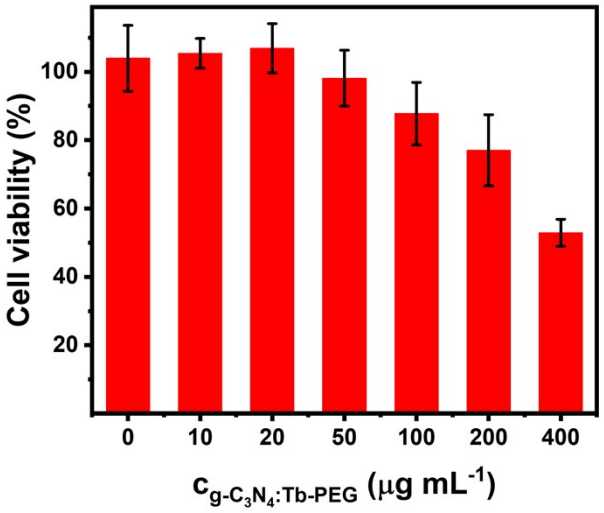


Figure S11. Cell viability of HepG2 cells incubated with g-C_3N_4:Tb-PEG nanosheets at different concentrations.

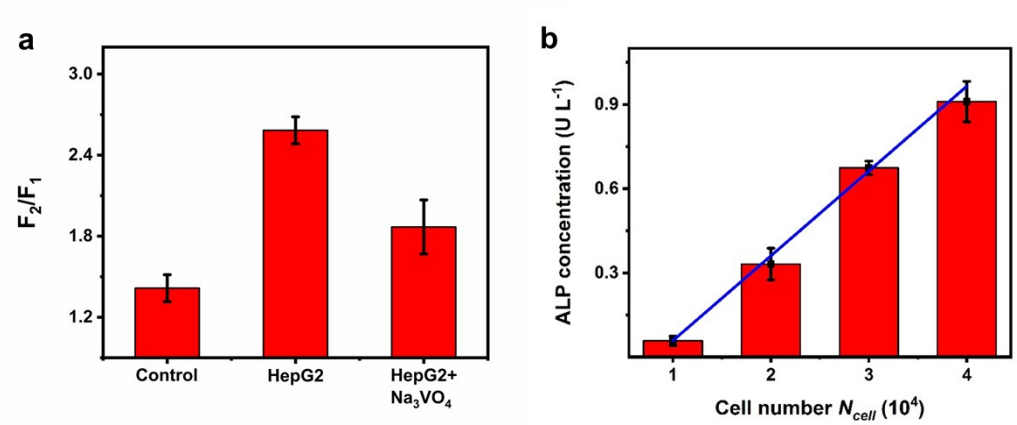


Figure S12. (a) Ratiometric fluorescence responses of the g-C_3N_4:Tb-PEG nanosheets based assay probe toward HepG2 cell lysates pre-treated without or with Na_3VO_4 serves as inhibitor. (b) The dependence of ALP concentration in HepG2 cell lysate on the number of HepG2 cells derived from the assay probe based on g-C_3N_4:Tb-PEG nanosheets.

Table S1 The quantum yields of g-C_3N_4:Tb and g-C_3N_4:Tb-PEG

λ_{ex} (nm)	λ_{em} (nm)	Quantum yield (%)	
		g-C_3N_4:Tb	g-C_3N_4:Tb-PEG
290	370	4.4±0.1	3.6±0.2
290	490	2.8±0.1	3.2±0.1
290	546	4.4±0.1	6.1±0.1

Table S2. Comparison of analytical performance using the ratiometric fluorescence assays for the detection of ALP activity.

Sensing platform	Sensing mechanism	Detection of ALP		Ref.
		Linear range (U/L)	Detection limit (mU/L)	
Phosphorylated chalcone derivative HCAP	ESIPT, AIE	0-150	150	2
Polymer dots (Pdots) + rhodamine B hydrazide (RB-hy)	FRET	0.005-15	1.8	3
1,8-naphthalimide derivative	ICT	0.5-5	380	4
B-CDs-MnO_2 + Y-CDs-MnO_2 +AAP	FRET	0.1-500	20	5
Ag_2S QDs + calcein + Ce^{3+}+PNPP	static quenching, AIE	2-100	1280	6
HBTP-mito	ESIPT	0-60	72	7
CdTe/CdS/ZnS/SiO_2 QDs + tyrosinase + phosphotyrosine + resorcinol	IFE	0.08-500	20	8
AuNCs + PNPP	IFE	0.2-5	30	9
UCNPs + OPD + Ag^+ + AAP	IFE	0.08-70	32	10
g-C_3N_4:Tb-PEG nanosheets + PNPP	IFE	0.010-10	2.2	This work

Table S3. Detection results of ALP in human serum by the ratiometric fluorescence approach with g-C_3N_4:Tb-PEG nanosheets as probe with comparison to those achieved by commercial alkaline phosphatase assay kit (colorimetry).

Serum	Spiked (U/L)	Found by ratiometric fluorescence (U/L)	Recov (%)	Found by commercial kit (U/L)	P value (t-test)
1	-	57.1±3.5	-	61.2±4.3	0.35
	100.0	160.8±4.5	103.7	-	
2	-	85.2±4.6	-	83.4±9.7	0.98
	100.0	184.9±6.7	99.6	-	
3	-	72.8±4.8	-	75.1±8.4	0.76
	100.0	170.4±3.6	97.6	-	

Table S4. Detection of ALP in livestock serum by the ratiometric fluorescence assay system and commercial kit method.

Serum	Spiked ($U\ L^{-1}$)	Found by ratiometric fluorescence ($U\ L^{-1}$)	Recovery (%)	Found by commercial kit ($U\ L^{-1}$)	P value (t-test)
Rabbit	-	43.7±1.5	-	40.8±1.7	0.21
	50.0	91.7±2.3	96.0	-	
Fetal	-	113.4±0.6	-	114.9±3.8	0.94
Bovine	50.0	166.1±2.1	105.4	-	

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