Fluorescence Resonance Energy Transfer-Based Nanoarchitectures for Monitoring Intracellular Deubiquitinating Enzyme Activity

Yan-Yan Liang[a 1], Jie Zhang[b,c1], Hui Cui[a], Zhen-Shu Shao[b], Chen Cheng[b], Yue-Bo Wang*[a], Huai-Song Wang*[b]

[a] *School of Chemistry and Chemical Engineering, Shandong University of Technology, Zibo 255000, China*

[b] *Department of Pharmaceutical Analysis, China Pharmaceutical University, Nanjing 210009, China*

[c] *Tianjin Key Laboratory of Food Biotechnology, Tianjin University of Commerce, Tianjin 300134, China*

* Corresponding Author:

ybwang@sdut.edu.cn (Yue-Bo Wang);

wanghuaisong@cpu.edu.cn; wanghuai1234@gmail.com (Huai-Song Wang)

[1] The first two authors contributed equally to this work.

Materials and reagents

Cetyltrimethylammonium bromide (CTAB), tetraethoxysilane (TEOS), glutathione (GSH), $TbCl_3 \cdot 6H_2O$, polyethylenimine (PEI), 3-(triethoxysilyl)propyl isocyanate were purchased Alfa Aesar. Adenosine triphosphate (ATP), 1,3-diphenyl-1,3-propanedione (DBM), human serum albumin (HSA), 1,10-Phenanthrolin-5-amine, 1-ethyl-3-(dimethyl-aminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxyl succinimide (NHS) were bought from Sigma-Aldrich (USA). UCH-L1 inhibitor was purchased from Aladdin. Ubiquitin-rhodamine (Ub-R) and UCH-L1 were purchased from Enzo Life Sciences, Inc.. All aqueous solutions were prepared from deionized water (18 $M\Omega \cdot cm^{-1}$). Unless otherwise stated, all chemical materials were purchased from commercial sources and used without further purification.

Apparatus and measurements

The morphologies of nanoparticles were characterized by scanning electron microscope (SEM, S-4800, Japan) and transmission electron microscopy (TEM, JEM-200CX, Japan). The X-ray photoelectron spectra (XPS) were tested on a PHI 5000 Versaprobe instrument (UlVAC-PHI, Japan). UV-vis absorption spectra were performed on a UV3600 spectrophotometer (Shimadzu, Japan). Fourier transform infrared (FTIR) spectra were collected on a Nicolet 6700 FTIR spectrometer (Thermo Scientific, USA). Fluorescence spectra were recorded on a RF-5301PC spectrophotometer (Shimadzu, Japan). Fluorescence lifetimes spectra were obtained on a FLS920 Fluorescence Lifetime and Steady State Spectrometer (Edinburgh, UK).

EXPERIMENTAL SECTION

Synthesis of Phen-Si.

Typically, 1,10-Phenanthrolin-5-amine (1.02 mmol) was added into chloroform (30 mL), and the mixture was ultrasonicated for 5 min. Then, 3-(triethoxysilyl)propyl isocyanate (1.7 mL) was added into the mixture. After stirred for 30 min, the chloroform was removed under vacuum at 25 ºC. The remaining yellow viscous mixture was stirred for another 20 h at 80 ºC. After the reaction, cold hexane was added to the mixture and a slightly yellow precipitate was obtained. The precipitate was then

dissolved in methanol and filtered. The methanol was removed by evaporation, and cold hexane was used to reprecipitate the production (Phen-Si). The prepared Phen-Si was dried at 60 ºC under vacuum.

Synthesis of mesoporous silica nanoparticles (MSNs).

In a typical synthesis procedure, CTAB (52 mg) was dissolved into 50 mL of H_2O/EtOH solution with volume ratio of 0 : 1, 1 : 1 or 1 : 4. After ultrasonicated for 10 min, $NH_3 \cdot H_2O$ solution (0.3 M) was added into the solution to adjust the pH value of the solution to 10.9. The solution was then stirred under 45 ºC, and 10 mL of TEOS ethanol solution, in which 1 mL TEOS has been dissolved in 48 mL ethanol, was added dropwise in 10 min. The reaction was performed for 1 h. Then milk-white MSNs were collected by centrifugation. For removing the surfactant, the MSNs were refluxed in a solution consisting of 30 mL of ethanol and 3 mL of hydrochloric acid under 75 ºC for 24 h. The MSNs were collected by centrifugation and washed 3 times with water and ethanol respectively. The final MSNs were dried for 6 h at 120 ºC in vacuum. The MSNs were named as MSN0/1, MSN1/1 and MSN1/4 respectively according to the reaction solvent.

Preparation of MSN-Tb.

The MSN1/4 nanoparticles (500 mg) were dispersed in a solution of dry dichloromethane (10 mL) containing Phen-Si (2 mg, 8 mg or 16 mg). The reaction was performed at 90 ºC for 12 h under a nitrogen atmosphere and stirring. Then, the prepared MSN-Phens were collected by filtration, rinsed with ethanol and dried under vacuum at 50 ºC. The prepared MSN-Phens were named as MSN-Phen1, MSN-Phen2 and MSN-Phen3 according to the amount of Phen-Si: 2 mg, 8 mg and 16 mg respectively.

For preparing MSN-Tbs, the prepared MSN-Phen1, MSN-Phen2 and MSN-Phen3 were respectively mixed with $TbCl_3 \cdot 6H_2O$ (20 mg) in 50 mL ethanol and refluxed at 80 ºC for 2 h under N_2 protection. Then, 1,3-diphenyl-1,3-propanedione (40 mg) was added into the suspension, and the reaction was carried out for another 24 h. After cooling, the precipitate was filtered. The prepared MSN-Tb1, MSN-Tb2 and MSN-Tb3 particles were washed with ethanol respectively, and dried in vacuum.

Modification of Ub-R on the surface of MSN-Tb2

Firstly, the PEI coating procedure was carried out by suspending 100 mg of MSN-Tb2 in PEI aqueous solution (10 wt %). The mixture was stirred at room temperature for 24 h. After PEI adsorption, the PEI modified MSN-Tb2 (MSN-Tb-PEI) was washed with deionized water.

The modification of Ub-R was carried out as follows: 0.1 mmol EDC and 0.25 mmol NHS were dissolved in 10 mL PBS (pH 7.4) containing 50 μM Ub-R. The mixture was stirred at 25 ºC for 20 min to activate the carboxylic group of Ub-R. Then, 50 mg MSN-Tb-PEI was dispersed in the solution, and stirred for 24 h at 25 ºC. The prepared MSN-Tb-UbR was collected by centrifuge at 12000 rpm, and re-dispersed in PBS (pH 7.4) for further experiment.

Fluorescent sensing

Typically, UCH-L1 with different concentrations was incubated with MSN-Tb-UbR (5 $mg \cdot mL^{-1}$) for 30 min at room temperature. The fluorescence spectra of the mixture were recorded.

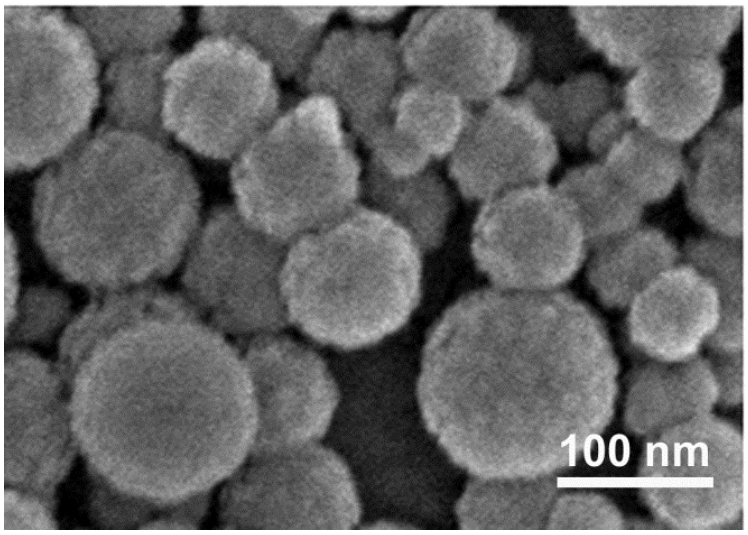
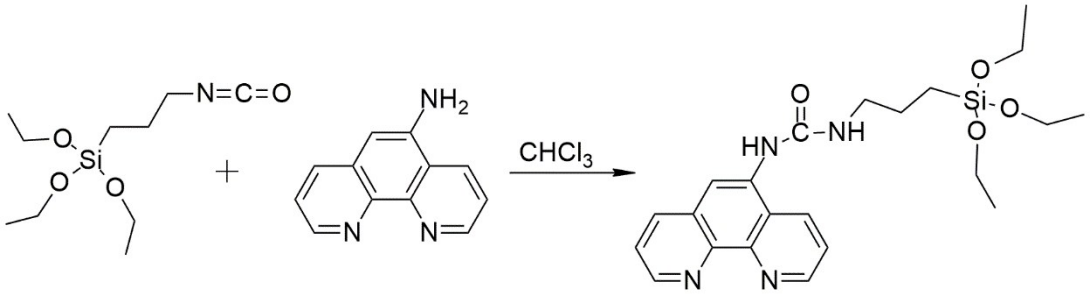


Fig. S1. SEM image of MSN1/4.



Scheme S2. Synthesis of Phen-Si.

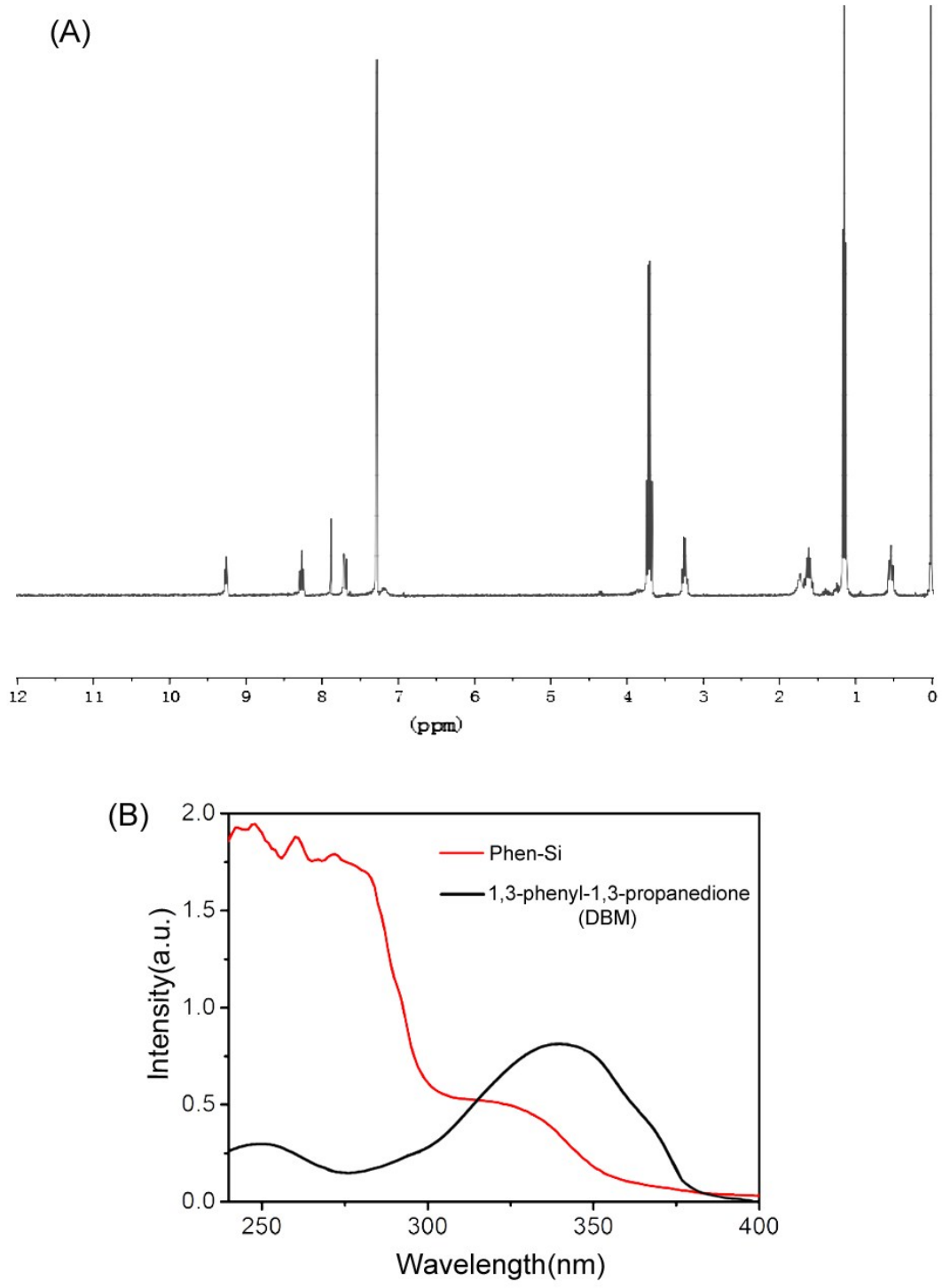


Fig. S3. (A) ^{1}H NMR ($CDCl_3$) spectrum of Phen-Si. In the ^{1}H NMR spectrum: δ = 0.54 (2H, m); 1.13 (9H, t); 1.62 (2H, m); 3.22 (2H, m); 3.69 (6H, q); 7.13 (2H, s); 7.70 (2H, m); 7.87 (1H, s); 8.25 (2H, m); 9.25 (2H, m). (B) UV absorbance spectra of Phen-Si and 1,3-diphenyl-1,3-propanedione (DBM).

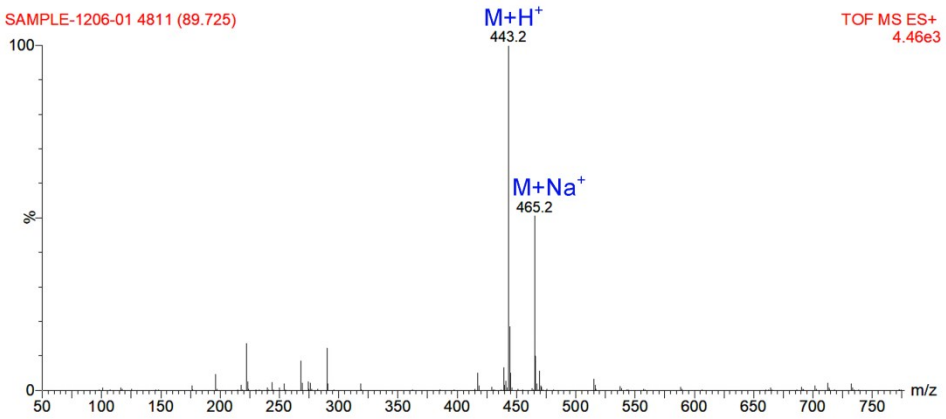


Fig. S4. ESI-TOF mass spectrum of Phen-Si. The calculated molecular weight of Phen-Si is: m/z 442.2 [M] ($C_{22}H_{30}N_4O_4Si$). The tested ESI-MS: m/z 443 $[M+H]^+$.

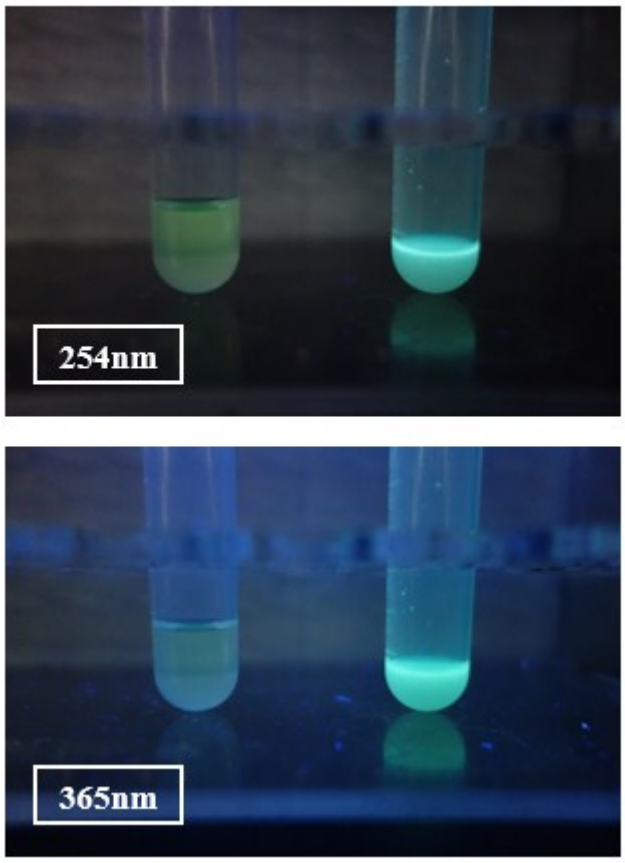


Fig. S5. Visible fluorescence of MSN-Tb2 and Phen-Tb irradiated under 365 nm light.

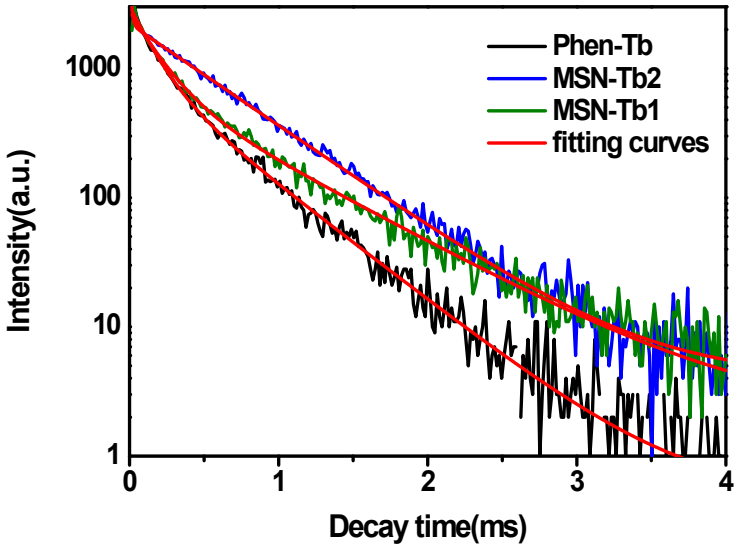


Fig. S6. Emission decay profiles of the MSN-Tbs.

Biexponential decay function obtained by fitting curve in Fig.S6 :

$$I = I_0 + A_1\exp(-t/\tau_1) + A_2\exp(-t/\tau_2) \qquad \text{(Eq. S1)}$$

where I is the luminescence intensity at time t, τ_1 and τ_2 are the decay time, A_1 and A_2 are constants.[1, 2]

The average lifetimes (τ_{av}) of each sample can be calculated by the formula：

$$\tau_{av} = (A_1\tau_1^2 + A_2\tau_2^2) / (A_1\tau_1 + A_2\tau_2) \qquad \text{(Eq. S2)}$$

Table S1. Time constants and average lifetime τ of Tb and MSN-Tbs, respectively.

Sample	τ_1(μs)	A_1	τ_2(μs)	A_2	I_0	τ_{av}(μs)
Tb	144.3	2093.62	485.3	981.81	0.4946	353.0
MSN-Tb1	694.5	787.91	176.3	2023.07	2.1158	490.0
MSN-Tb2	548.4	2219.13	9.6	4460.62	4.0655	530.1

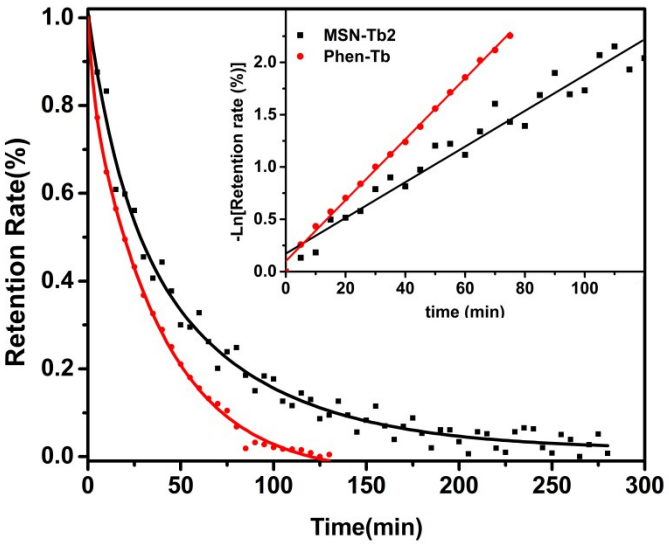


Fig. S7. Fluorescence intensity of Phen-Tb and MSN-Tb2 under UV light as a function of time.

Table S2. Kinetic parameters related to fluorescence stability. the first-order kinetics formula obtained according to the Langmuir–Hinshelwood (L–H) model .[3]

Sample	first-order kinetic equation	Rate constant (min^{-1})	R^2
MSN-Tb2	Y=0.0171X+0.1724	0.0171	0.9571
Phen-Tb	Y=0.0291X+0.1007	0.0291	0.9974

$$R_0 = \left(\frac{9000 \times \ln(10) k^2 Q_D}{128 \pi^5 n^4 N_A} J \right)^{1/6} \quad \text{(Eq. S3)}$$

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \quad \text{(Eq. S4)}$$

Where k^2 is the dipole orientation factor, usually assumed to be 2/3.[4] n is the refractive index of the medium, 1.4 for biomolecules in aqueous solution.[5] Q_D is the fluorescence quantum yield of the donor (Q_D= 0.36). J is the spectral overlap integral. We calculate the value of J, which is 4.6×10^{-14} $M^{-1} \cdot cm^3$. N_A is Avogadro's number. Also, F and F_0 are the fluorescence intensities in the presence and absence of Ub-Rs, respectively. r is the binding distance between donor and receptor.

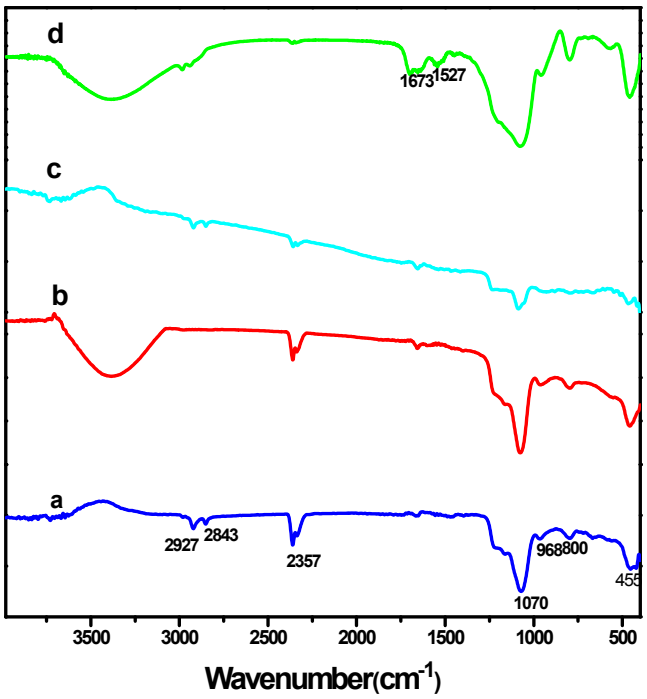


Fig. S8. FT-IR spectra of (a) MSN-CTAB, (b) MSN, (c) MSN-Phen2, (d) MSN-Tb2.

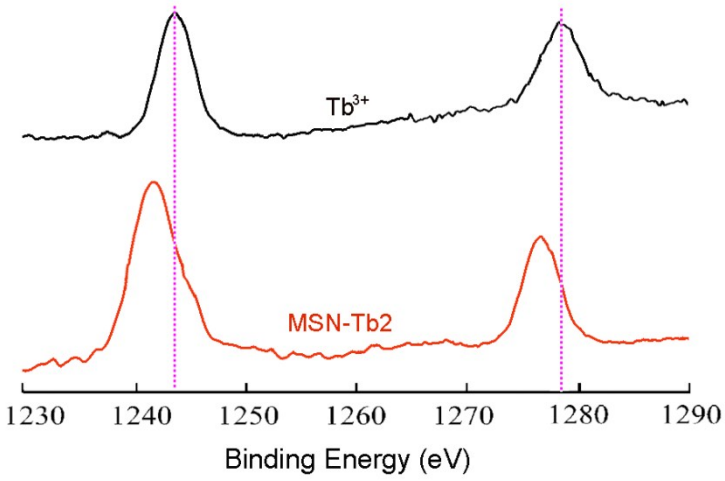


Fig. S9. XPS spectra of $TbCl_3$ and MSN-Tb2.

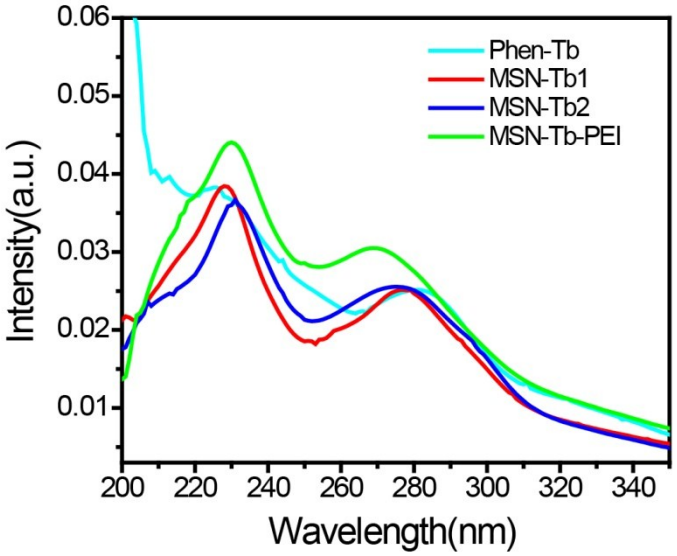


Fig. S10. UV-visible absorbance spectra of the products.

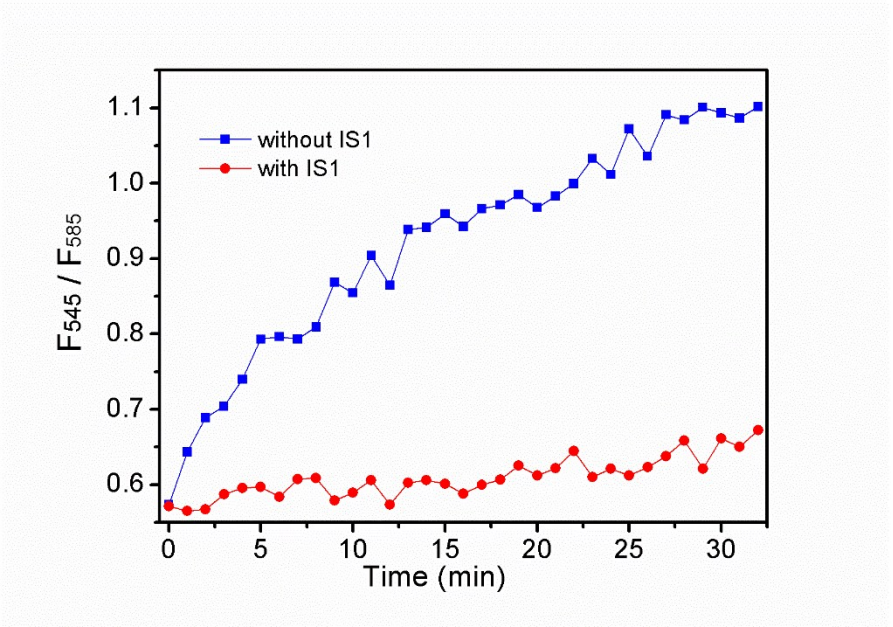


Fig. S11. Progress curve for the UCH-L1-catalyzed hydrolysis of MSN-Tb-UbR with and without IS1. The UCH-L1 concentration: 20 nM; the IS1 concentration: 25 μM; and the MSN-Tb-UbR concentration: 5 $mg \cdot mL^{-1}$.

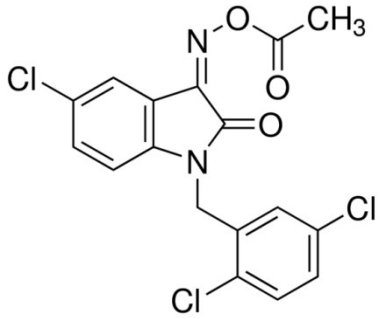


Fig. S12. UCH-L1 inhibitor IS-1 (an isatin *O*-acyl oxime compound)

References

1. J. Wu, S. Shi, X. Wang, J. Li, R. Zong and W. Chen, J. Mater. Chem. *C*, 2014, **2**, 2786-2792.
2. G. B. Piland, J. J. Burdett, D. Kurunthu and C. J. Bardeen, *J. Phys. Chem. C*, 2013, **117**, 1224-1236.
3. X. Cheng, H. Liu, Q. Chen, J. Li and P. Wang, J. Hazard. Mater., 2013, **254-255**, 141-148.
4. Z. Li, Z. li, L. Yang, Y. Xie, J. shi, R. Wang and J. Chang, J. Fluoresc., 2015, **25**, 451-463.
5. Y. Chang, C. Cai, L. Li, J. Miao, E. Ucakturk, G. Li, M. Ly and R. J. Linhardt, Anal. Chem., 2013, **85**, 9356-9362