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Supporting Information

A pH and Temperature Dual Responsive Polymeric Fluorescent Sensor and Its Imaging in Living Cells

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1. General information

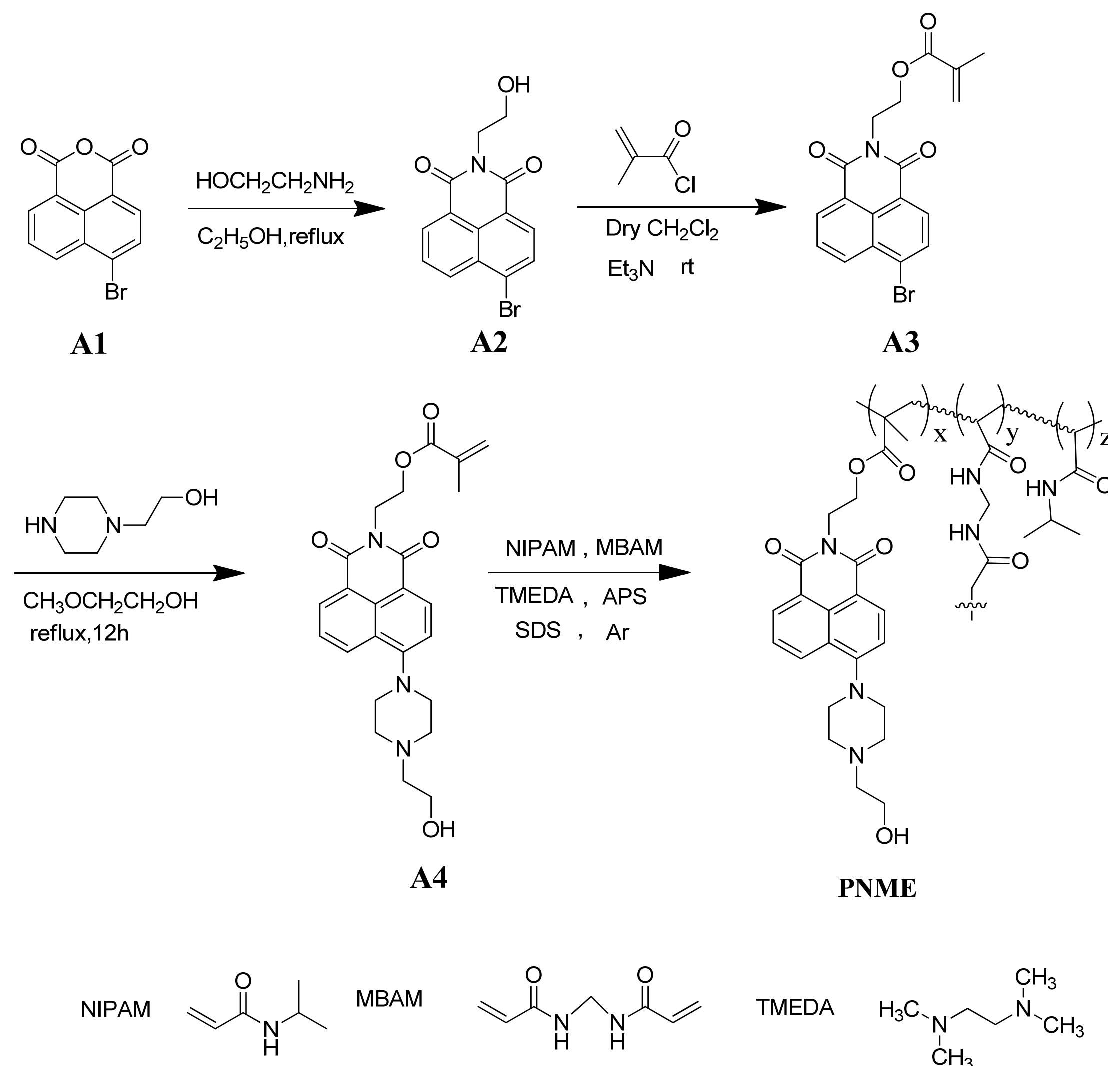
1.1. Materials. All the reagents used were of analytical grade, which were purchased from Aladdin reagent Co without further purification except especial instruction. *N*-isopropylacrylamide (NIPAM) monomer was used after recrystallization from n-hexane. Ammonium persulfate (APS), Sodium docecyl sulfate (SDS), *N, N'*-methylenebisacrylamide (MBAM) were purchased from Aladdin reagent Co.

All the organic solvents were of analytical grade. Dichloromethane and Et_3N were distilled by CaH_2 to remove the water before used. Water was purified by a Milli-Q system.

LysoTracker Red (Cat. L7528) was purchased from Invitrogen. Dulbecco's Phosphate Buffered Saline (DPBS) powder (Cat. SH30013.04), DMEM (High glucose) medium (Cat. SH30243.01B) were from Hyclone. Foetal Bovine Serum (FBS) (Cat. SFBS) was from Bovogen.

1.2. Instruments and Measurements. The transmission electron microscopy (TEM) image was obtained by a JEM-2010HT (200 keV) transmission electron microscopy. The hydrodynamic diameter was estimated by dynamic light scattering (DLS) measurements with a Zetasizer Nano ZS (Malvern Instruments). Nuclear magnetic resonance (NMR) spectra were measured on a Bruker AM-400 spectrometer with chemical shifts reported in ppm (TMS as internal standard). Mass spectra were measured on a HP 1100 LC-MS spectrometer. Fluorescence spectra were determined on a Varian Cary Eclipse Fluorescence Spectrophotometer with a temperature controller. Absorption spectra were determined on a Varian Cary Bio100 UV-Visible Spectrophotometer. All pH measurements were made with a Sartorius basic pH-Meter PB-10. The images of subcellular localization of PNME were used by Olympus FV1000 + IX481 confocal laser scanning microscopy using UPLSAPO 100X oil objective (NA: 1.40). The integrated images of HeLa cells incubated with PNME at different temperature were used by Nikon A1R confocal laser scanning microscope using Plan Apochromat violet corrected (VC) 40X oil objective.

2. Experimental section



Scheme S1. Synthetic route of polymeric fluorescent sensor **PNME**

2.1. Synthesis of monomer A4

The synthesis route was depicted in Scheme S1. The procedures are as follows:

6-bromo-2-(2-hydroxyethyl)-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (A2)

6-bromobenzo[*de*]isochromene-1,3-dione (A1) (500.0 mg, 1.8 mmol) and 2-aminoethanol (0.1 mL, 1.8 mmol) were dissolved in 20.0 mL ethanol. The reaction mixture was heated to reflux for 1 h. Then, the solution was cooled to room temperature, precipitated, filtered, and dried. The 6-bromo-2-(2-hydroxyethyl)-1*H*-benzo [*de*]isoquinoline-1,3(2*H*)-dione was obtained as a white solid (500.0 mg, 87%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.70 (d, *J*=7.2 Hz, 1H, Ar-H), 8.62 (d, *J*=8.4 Hz, 1 H, Ar-H), 8.45 (d, *J*=7.6 Hz, 1H, Ar-H), 8.08 (d, *J*=8.0 Hz, 1H, Ar-H), 7.89 (t, *J*=7.6 Hz, 1H, Ar-H), 4.48 (t, *J*=4.8 Hz, 2H, CH_2OH), 4.01 (t, *J*=4.8 Hz, 2H, NCH_2).

2-(6-Bromo-1, 3-dioxo-*1H*-benzo[*de*]isoquinolin-2(*3H*)-yl)ethyl methacrylate (A3)

6-bromo-2-(2-hydroxyethyl)-1*H*-benzo[*de*]isoquinoline-1, 3(2*H*)-dione (A2) (500.0 mg, 1.6 mmol) was dissolved in dry dichloromethane, and Et_3N(1.7 mL, 12.5 mmol) was added. The solution was cooled to 0 °C and methacryloyl chloride (1.3 g, 12.5 mmol) dissolved in 10.0 mL dry dichloromethane was added from a dropping funnel over a period of 1 h at 0 °C. Then removed the ice bath, the reaction was continue 3 h at room temperature. Then, the solution was poured to the ice water, and extracted by dichloromethane (50 mL × 3). The organic phase was dried over Na_2SO_4, and concentrated by evaporation. The raw product was purified by column chromatography on silica gel using petroleum ether / ethyl acetate (15:1, volume) as an eluent to obtain target compound **A3** as a white solid (540.0 mg, 89%).

^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.64 (d, *J*=7.2 Hz, 1H, Ar-H), 8.61 (d, *J*=8.4 Hz, 1H, Ar-H), 8.45 (d, *J*=7.6 Hz, 1H, Ar-H), 8.08 (d, *J*=8.4 Hz, 1H, Ar-H), 7.88 (t, *J*=7.6 Hz, 1H, Ar-H)，6.06 (s, 1H, C=CH_2), 5.30 (s, 1H, C=CH_2), 4.57 (t, *J*=5.2 Hz, 2H, CH_2OCO), 4.51 (t, *J*=5.2 Hz, 2H, NCH_2), 1.88 (s, 3H, CH_3).

2-(6-(4-(2-hydroxyethyl)piperazin-1-yl)-1,3-dioxo-*1H*-benzo[*de*]isoquinolin-2(*3H*)-yl)ethyl methacrylate (A4)

2- (6- bromo-1, 3-dioxo-1*H*-benzo [*de*] isoquinolin-2 (3*H*)-yl) ethylmethacrylate) (100.0 mg , 0.3 mmol), 2 -(piperazin-1-yl)ethanol (72.0 mg , 0.5 mmol), and potassium iodide (30.0 mg) were dissolved in 15.0 mL 2-methoxyethanol. Then, the solution was refluxed for 12 h under the Argon atmosphere. The solvent was evaporated under vacuum and the crude product was purified by column chromatography on silica gel using dichloromethane/methanol (30/1, volume) as an eluent to obtain target compound **A4** as a yellow powder (24.0 mg, 21%). The target compound was confirmed by ^{1}H NMR, ^{13}C NMR, HRMS.

^{1}H NMR (400 MHz, $CDCl_3$, TMS, 25 °C) δ 8.61 (d, *J*=7.2 Hz, 1H, Ar-H), 8.5 5 (d, *J*=8.0 Hz,1H, Ar-H), 8.44 (d, *J*=8.4 Hz, 1H, Ar-H), 7.72 (t, *J*=7.6 Hz, 1H, Ar-H), 7.25 (d, *J*=8.0 Hz, 1H, Ar-H), 6.07 (s, 1H, C=CH_2), 5.62 (s, 1H, C=CH_2), 4.57 (t, *J*=5.2 Hz, 2H, CH_2OCO), 4.50 (t, *J*=5.2 Hz, 2H, NCH_2), 3.75 (t, *J*=5.2 Hz, 2H, CH_2OH), 3.35 (br, 4H， piperazin), 2.90 (br, 4H, piperazin), 2.76 (t, *J*=5.2 Hz, 2H, piperazin-CH_2), 1.89 (s, 3H, CH_3),

^{13}C NMR (100 MHz , $CDCl_3$, TMS, 25 °C) δ 18.3, 38.8, 52.8, 53.0, 57.7, 59.5, 62.1, 115.1, 116.8, 123.1, 125.8, 126.2, 130.0, 130.3, 131.3, 132.7, 136.1, 155.7, 163.9, 164.4, 167.3.

HRMS (ESI): Calcd for ($M+H^+$), 438.1951; Found, 438.2027.

2.2. Preparation of polymeric fluorescent sensor PNME

PNME was synthesized according to the reported procedure previously with minor alteration. The procedures are as follows:

N-isopropylacrylamide (NIPAM, 2.0 mmol), *N*, *N'*-methylenebisacryl-amide (MBAM, 20.0 μmol), N-(2-methacryloxyethyl)-4-(4-（2-hydroxyethyl）piperazine-1-yl)-1, 8-naphthalimde (40.0 μmol) and *N*, *N*, *N'*, *N'*-tetramethylethylenediamine (TMEDA, 58.0 μmol) were dissolved in 30 mL of sodium dodecyl sulfate solution (SDS, 12.6 mM). Then the solution was drained by Argon for 30 min to remove the dissolved oxygen. Next, ammonium persulfate (APS, 560.0 μmol) was added to the above solution to initiate the polymerization. The mixture was stirred by magneton at 70 ^{o}C for 4 hours under Argon atmosphere. Then, the mixture was poured into 100.0 mL water and ammonium chloride was added to make the nanogels precipitated by a salting out technique. The resulting solution was purified by dialysis and dried by freeze-dried. **PNME** was obtained as light yellow floc-like material.

2.3 Cell culture and imaging

2.3.1 The lysosome imaging

Cell Culture. HeLa cells were obtained from American Type Culture collection, and grown in Dulbecco's modification of Eagle's medium Dulbecco (DMEM/high: with 4500 mg/L Glucose, 4.0 mM L-Glutamine, and 110 mg/L Sodium Pyruvate) supplemented with 10% foetal bovine serum (FBS). Cells were incubated in a 5% CO_2 humidified incubator at 37 ^{o}C and typically passaged with sub-cultivation ratio of 1:4 every two days.

***In situ* Imaging.** HeLa cells, pre-washed twice, were labeled with 0.28 mg/mL **PNME** in DPBS (containing 1% DMSO) at 37 ^{o}C. After 30 min, the cells were washed to remove unbounded probes for six times before *in situ* imaging by Olympus FV1000 + IX481 confocal laser scanning microscopy using UPLSAPO 100X oil objective (NA: 1.40), with excitation by 405 nm laser, and 500-600 nm emission light was collected.

Co-localization of probes with LysoTracker Red. For **PNME** co-localization with LysoTracker Red: HeLa cells were pre-washed twice, and incubated with 0.28 mg/mL **PNME** in DPBS with 1% DMSO for 30 min at 37 ^{o}C. After the removal of unbounded probes by washing for six times, the cells were then labeled by 100 nM LysoTracker Red for 30 min at 37 ^{o}C. Cells were washed twice before observation under confocal microscopy in DPBS.

The excitation of **PNME** is 405 nm, and 500-600 nm emission light was collected. The excitation of LysoTracker Red is 635 nm, and > 650 nm emission light was collected.

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2.3.2 The intracellular temperature imaging

The Hela cells were seeded to 35 mm glass bottom dishes the day before imaging to get the cell density about 80%. The cells were labeled with 0.28 mg/mL PNME in DPBS buffer at 37 ^{o}C. After 30 min, the cells were washed for six times to remove unbounded probes using DPBS buffer at 25 ^{o}C before in situ imaging with a Nikon A1R confocal laser scanning microscope using Plan Apochromat violet corrected (VC) 40X oil objective, with excitation by 405 nm laser, and 500-550 nm emission light was collected. The images at 25 ^{o}C were taken after the cells were incubated in the cell culture accessories of the microscopy at 25 ^{o}C for 20 min. Then, the setting temperature of the incubator was increased to 37 ^{o}C, after 20 min, the temperature was reached, then, the images of the cells were taken in the same dish at 37 ^{o}C.

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3. Results

3.1 Characterization of polymeric fluorescent sensor PNME

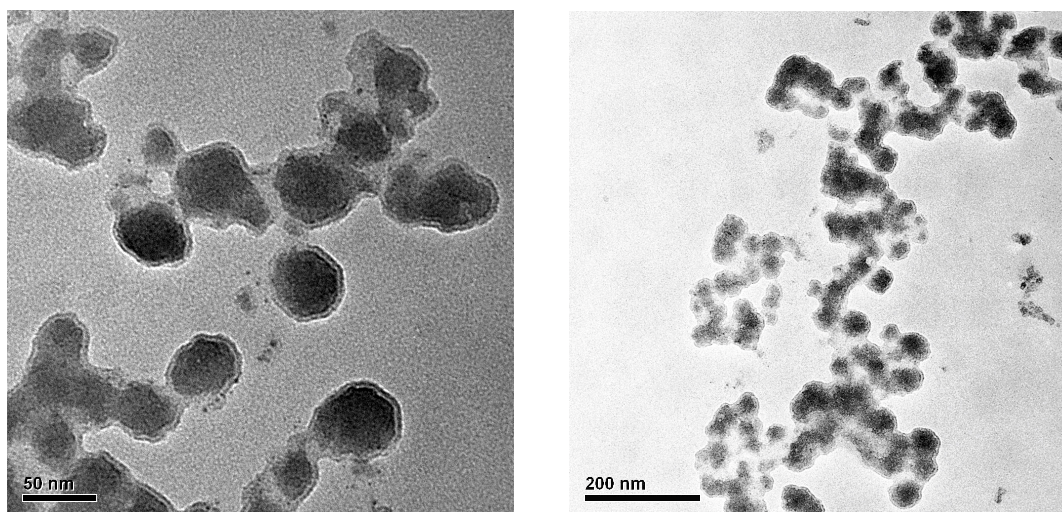


Fig. S1. A transmission electron microscopy (TEM) image of polymeric fluorescent sensor **PNME**

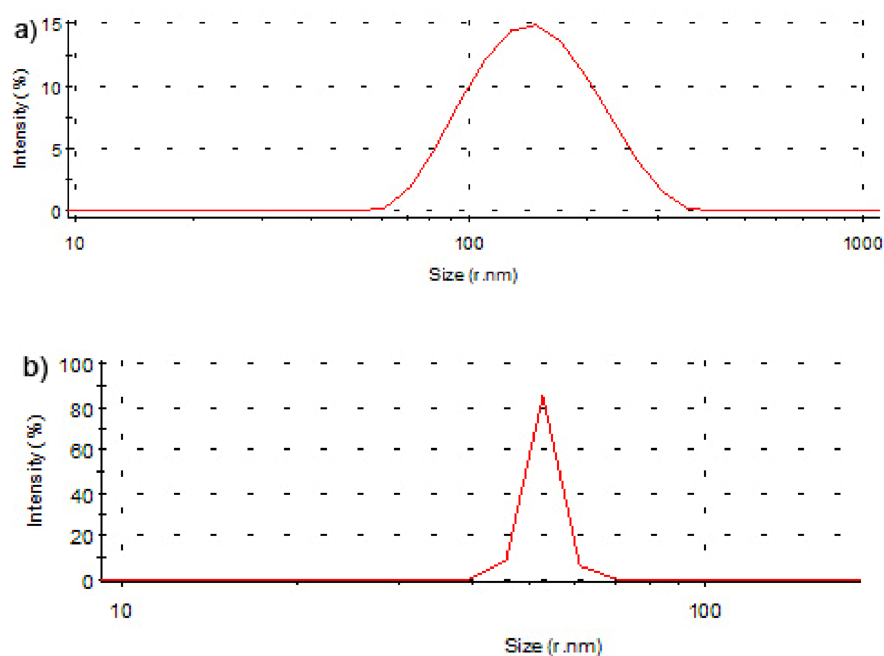


Fig. S2. Size distribution of polymeric fluorescent sensor **PNME** examined by dynamic light scattering measurements in water. a) 25 °C, b) 40 °C.

3.2 pH dependence of fluorescence response of polymeric sensor PNME (1 mg mL^{-1}) at different temperature.

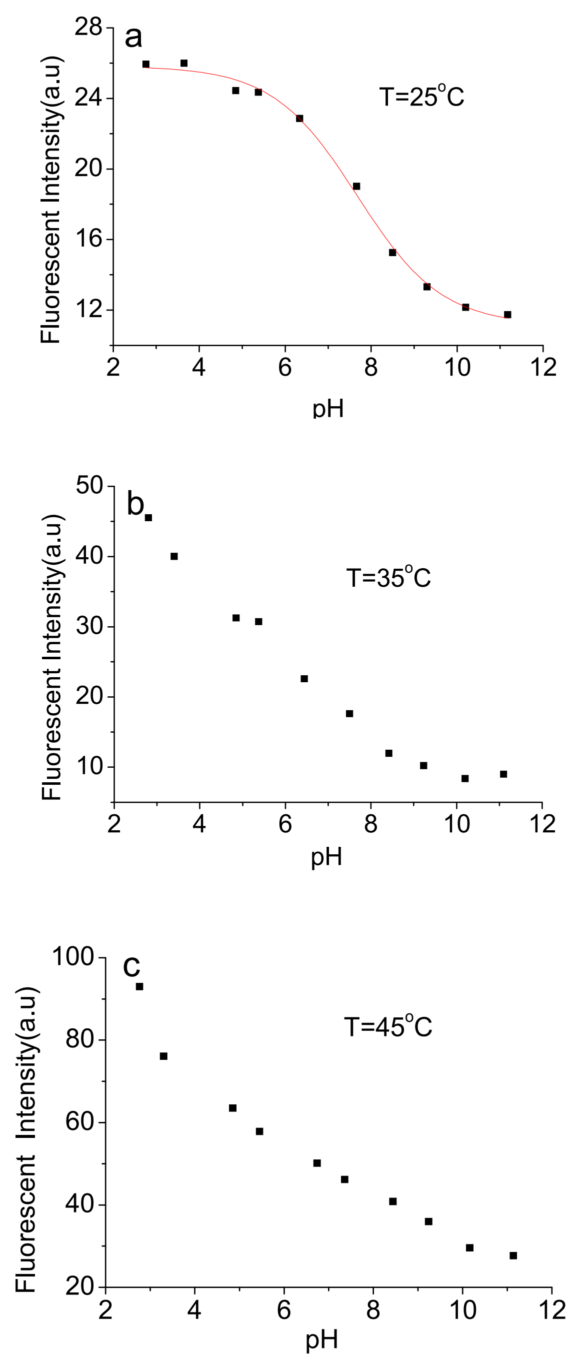


Fig. S3. pH dependence of fluorescence response of polymeric sensor **PNME** (1 mg mL^{-1}) at different temperature, (a) 25 °C; (b) 35 °C; (c) 45 °C. Excitation wavelength: 410 nm.

3.3 Fluorescence spectra change of the polymeric fluorescent sensor PNME at various pH values measured in water at 25 °C

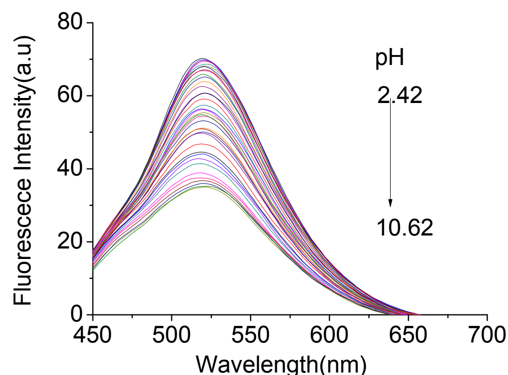


Fig. S4. Fluorescence spectra change of the polymeric fluorescent sensor **PNME** (7 mg mL^{-1}) at various pH values measured in water at 25 °C, Excitation wavelength: 410 nm.

3.4 Temperature dependence of fluorescence response of polymeric sensor PNME (1 mg mL^{-1}) at different pH values.

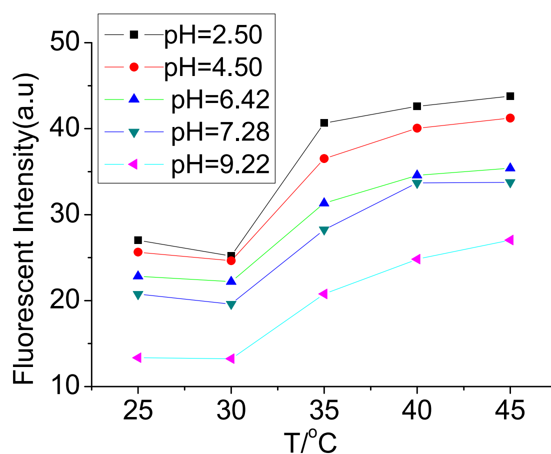


Fig. S5. Temperature dependence of fluorescence intensity of polymeric sensor **PNME** (1 mg mL^{-1}) at different pH values. Excitation wavelength: 410 nm.

3.6 Fluorescence spectral change of the polymeric fluorescent sensor PNME at different temperature measured in water at pH 7.08.

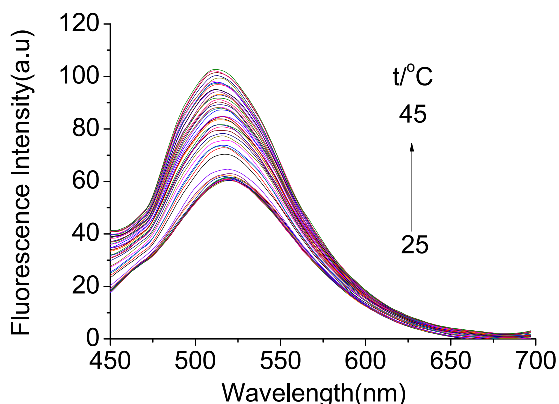


Fig. S6 Fluorescence spectral change of the polymeric fluorescent sensor **PNME**(7 mg mL^{-1}) at different temperature measured in water at pH 7.08. Excitation wavelength: 410 nm.

3.7 Fluorescence spectra change of A4 at various pH values measured in water at 25 °C

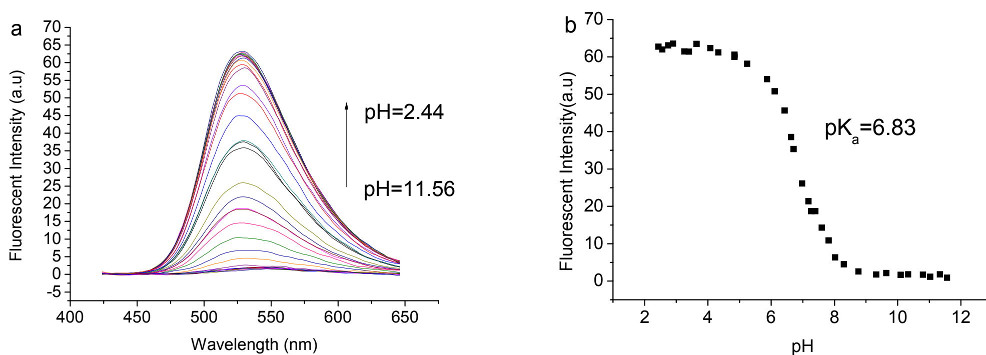


Fig. S7 (a) Fluorescence spectral change of the fluorescent sensor **A4** (10 μM) at various pH values measured in water at 25 °C (b) Fluorescence response to pH variation at 25 °C. Excitation wavelength: 410 nm.

3.8 The fluorescence intensity of A4 in different solvents

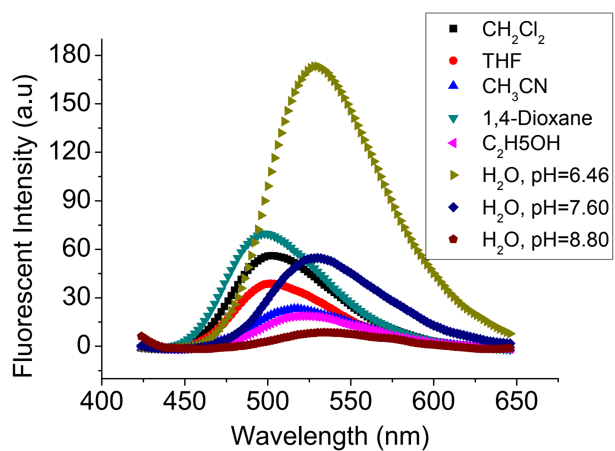


Fig. S8 The fluorescence intensity of A4 (10 μM) in different solvents. Excitation wavelength: 410 nm.

3.9 The ratio of (x/y/z) of A4/MBAM/NIPAM units in PNME

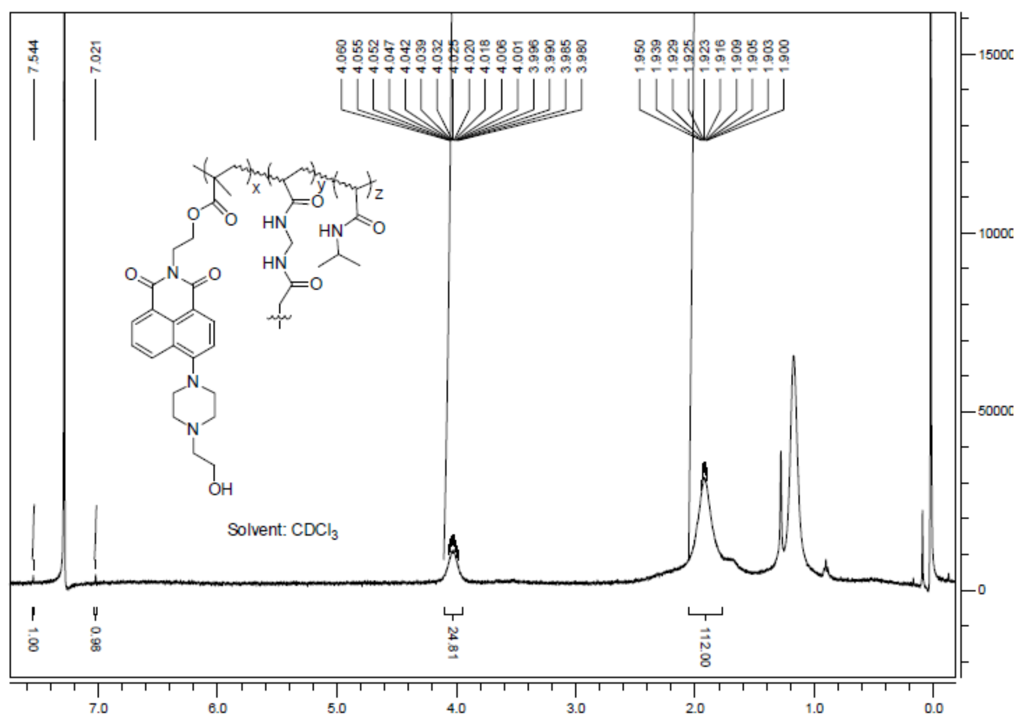


Fig. S9 the ^{1}H-NMR spectrum of PNME

As shown in Fig. S9, the peaks of 7.544 ppm and 7.021 ppm are assigned to aromatic protons, and the peaks between 3.980 and 4.060 ppm are assigned to the monomer of MBAM(NHCH_2NH), also, the peaks between 1.900 and 1.950 ppm are assigned to the monomer of NIPAM(CH(CH_3)$_2$). The ratio (x/y/z) of A4/MBAM/NIPAM units in **PNME** was 0.7/18.0/81.3 by caculation.

4. The charaterization of monomer A4

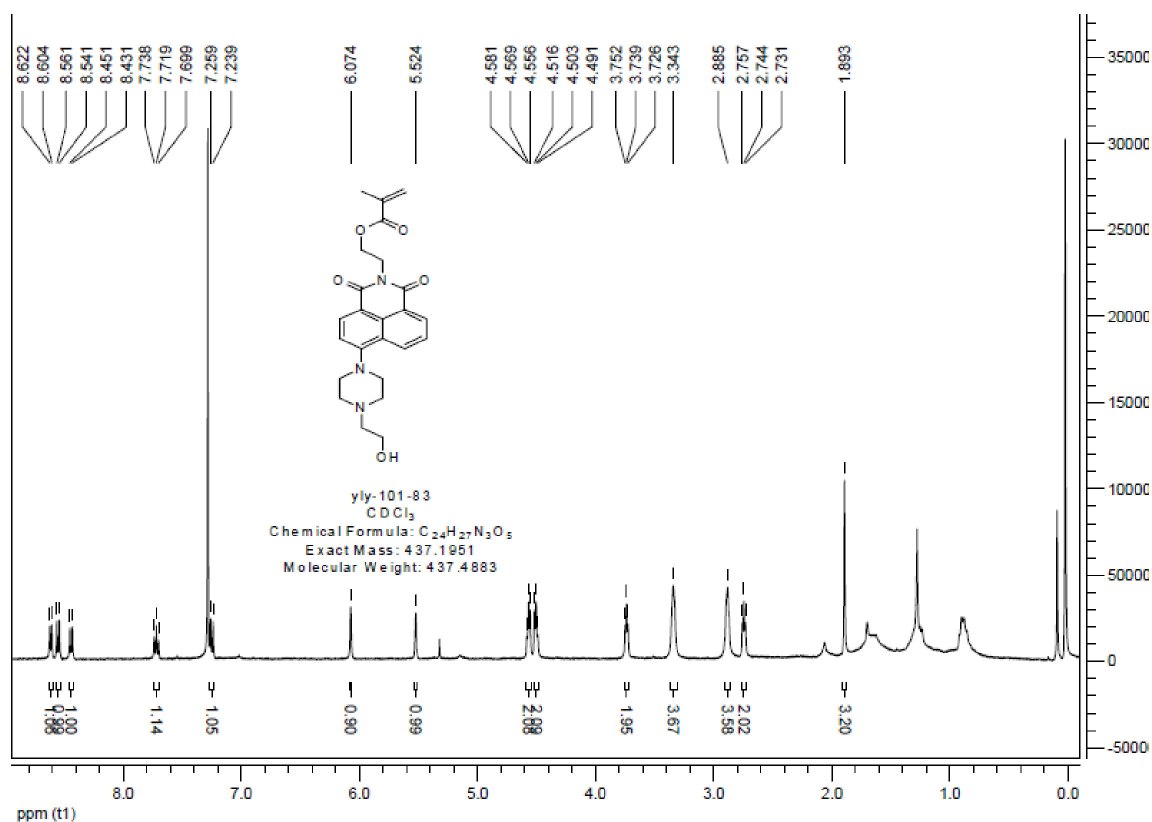


Fig. S10 the ^{1}H-NMR spectrum of A4

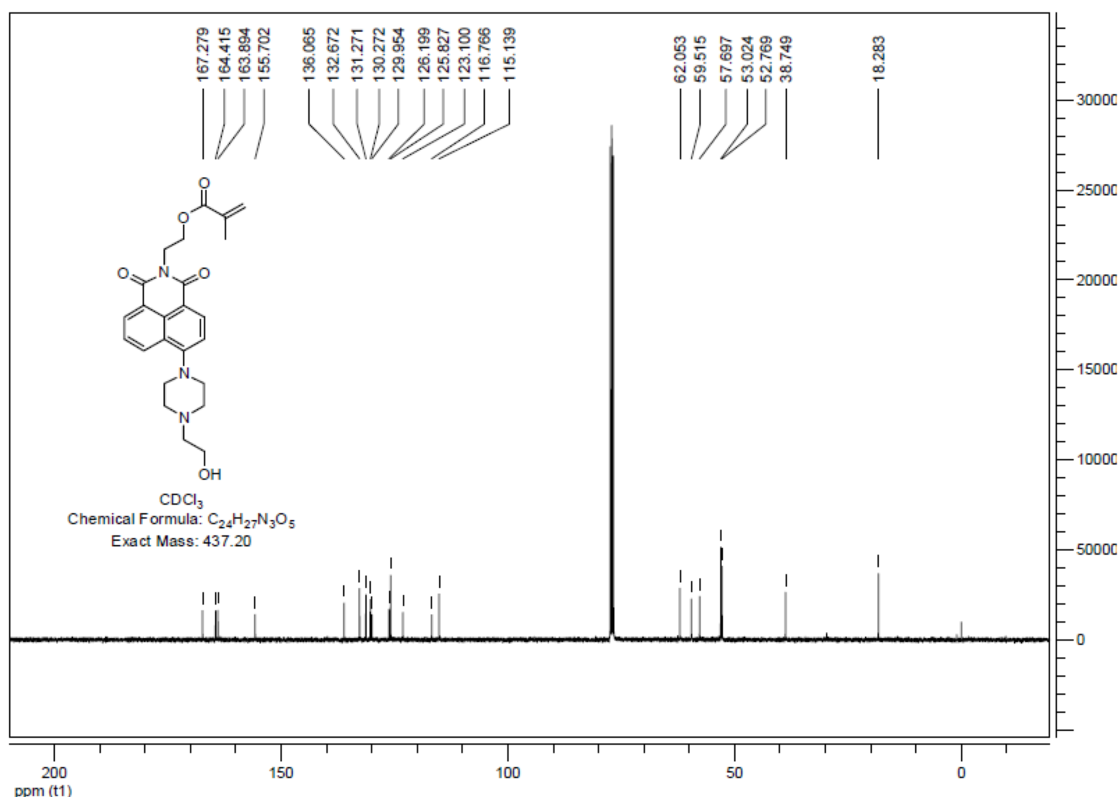


Fig. S11 the ^{13}C-NMR spectrum of A4

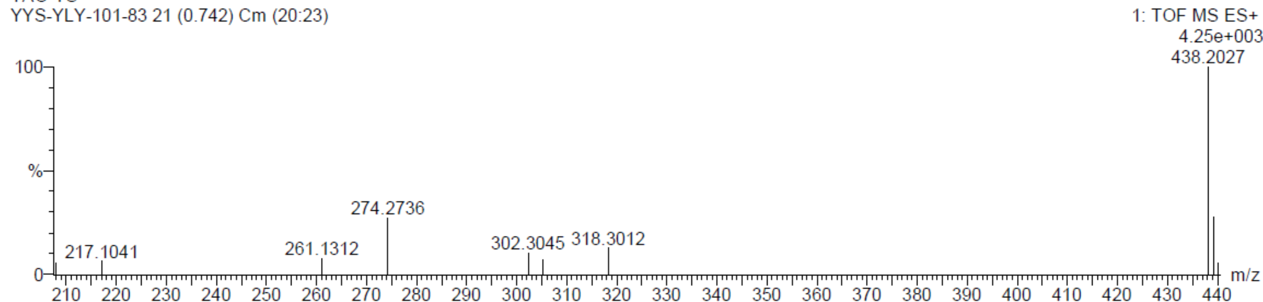
Elemental Composition Report

Page 1

Single Mass Analysis

Tolerance = 3.0 mDa / DBE: min = -1.5, max = 100.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 2

Monoisotopic Mass, Even Electron Ions
518 formula(e) evaluated with 2 results within limits (up to 1 best isotopic matches for each mass)
Elements Used:
C: 0-32 H: 0-50 N: 0-6 O: 0-20
YAO-YS
YYS-YLY-101-83 21 (0.742) Cm (20:23)



Minimum:				-1.5			
Maximum:		3.0	50.0	100.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
438.2027	438.2029	-0.2	-0.5	12.5	7.0	0.0	C24 H28 N3 O5

Fig. S12 the HR-MS spectrum of A4

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5. References

1. Gota, C.; Okabe, K.; Funatsu, T.; Harada, Y.; Uchiyama, S. *J. Am. Chem. Soc.* 2009**, 131** (8), 2766-2767.
2. Pietsch, C.; Hoogenboom, R.; Schubert, U. S. *Angew. Chem.Int.Ed*. 2009, **48** (31), 5653-5656.
3. Uchiyama, S.; Makino, Y. *Chem. Commun*. 2009, (19), 2646-2648.
4. Wang, D. P.; Miyamoto, R.; Shiraishi, Y.; Hirai, T. *Langmuir*. 2009, **25** (22), 13176-13182.
5. Tian, Y. Q.; Su, F. Y.; Weber, W.; Nandakumar, V.; Shumway, B. R.; Jin, Y. G.; Zhou, X. F.; Holl, M. R.; Johnson, R. H.; Meldrum, D. R. *Biomaterials.* 2010, **31** (29), 7411-7422.