Nanomolar detection of adenosine triphosphate (ATP) using a nanostructured fluorescent chemosensing ensemble

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Experimental Details

1,8-bis(bromomethyl)naphthalene (1), N-(pyridin-3-yl)acetamide (Py-1) and N-(pyridin-3-yl)tetradecanamide (Py-13) were prepared according to a literature method.¹,² NMR spectra were recorded using a Varian instrument (400 MHz). Chemical shifts were expressed in ppm and coupling constants (J) in Hz. UV-vis spectra were recorded on a Hitachi UV-3010 spectrophotometer. Fluorescence spectra were recorded on a Hitachi F-4500 fluorescence spectrophotometer. The particle size of the aggregation was measured using a Zetasizer Nano-ZS90 instrument. Cellular imaging was recorded on a Zeiss LSM 710 laser scanning confocal microscopy. Fluorescence quantum yields and fluorescence lifetimes were measured using an Edinburgh-steady-state/transient fluorescence spectrometer FLS 1000. Fluorescence imaging of C. elegans was carried out using a Leica biological microscope DM 3000.

Calculation of binding constant and detection limit

The binding constants between hosts and guests were obtained by the fluorescence titration data. According to the Benesi-Hildebrand method, the equation for host:guest (1:n) complex is given below:
In the equation, $I_0$ is the fluorescence intensity of the free probe, $I$ is the intensity in which a certain guest is added, $I'$ is the strength in which the guest is saturated, and $K$ is the binding constant.

The detection limit (DL) is calculated as follows:

$$DL = \frac{3\sigma}{K}$$

Where $K$ is the slope of the linear fit and $\sigma$ is the standard deviation of 11 blank measurements.

**Cytotoxicity Assay by MTT Method**

First, mouse liver cancer cells Hepa1-6 were cultured and 20,000 cells were seeded in a 96-well microtiter plate before the assay. Then the cells were treated with various concentrations of the PyNp-C13/UD assembly (0−40 μM) at 37 °C for 24h. Afterwards the medium was discarded, 100 μL of freshly prepared 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 1 mg/mL in PBS buffer solution) was added to each well. The wells were incubated for a further 4 h. The number of the alive cells was expressed as follows:

$$\text{Percent viability} = \frac{A_{570} \text{(Treated cells)} - \text{background}}{A_{570} \text{(Untreated cells)} - \text{background}} \times 100\%$$

where $A_{570}$ represents the absorbance at 570 nm.

**Cell culture and fluorescence imaging**

Mouse liver cancer Hepa1-6 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum (Invitrogen Corp., Carlsbad, CA) and penicillin (100 units/mL)-streptomycin (100 μg/mL) (Invitrogen Corp., Carlsbad, CA) at 37 °C in a humidified incubator containing 5% CO$_2$ in air. The cells were pretreated with NaN$_3$ (5 mM) or Ca$^{2+}$ (5 mM) for 1 h. Then, the cells were incubated with DMEM containing 10% PBS and 5 μL of complex probe PyNp-C13/UD (12 μM/6 μM) at 37 °C for 3h, washed twice with PBS, and mounted on the
microscope stage. Fluorescence images were captured by a Zeiss LSM 710 confocal laser-scanning microscope equipped with a live cell workstation.

**In vivo C. elegans imaging**

*C. elegans* were washed from their solid medium into a 20 mL centrifuge tube using an M9 buffer solution and centrifuged to settle the nematodes, followed by dispensing the nematodes into three sets of 2 mL small centrifuge tubes. One group of *C. elegans* was added to PyNp-C13/UD (40 µM/20 µM) for 3 h at 20 °C; another group was treated with ATP (100 µM) for 4 hours at 20 °C and then incubated with PyNp-C13/UD (40 µM/20 µM) for 3 h. The pretreated *C. elegans* were then anesthetized and washed with M9 buffer solution. Fluorescence images of the nematodes were recorded using a Leica biological microscope DM 3000.

![Scheme S1 Synthesis route of PyNp-C13 and PyNp-C1.](image)

**Synthesis of PyNp-C13**

First, compounds 1 (300 mg, 0.96 mmol) and Py-13 (200 mg, 0.67 mmol) were dissolved in acetonitrile (40 mL) under a nitrogen atmosphere. The stirred solution was heated and refluxed overnight. The solvent was evaporated *in vacuo* and the crude residue was purified by column chromatography (CH₂Cl₂/MeOH, 95: 5, v/v) to
obtain PyNp-C13 as a white solid (240 mg, yield 46%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ ppm: 11.08 (s, 2H), 9.43 (s, 2H), 8.74 (d, J = 5.8 Hz, 2H), 8.51 (d, J = 8.6 Hz, 2H), 8.21-8.07 (m, 4H), 7.56 (t, J = 7.6 Hz, 2H), 6.93 (d, J = 7.4 Hz, 2H), 6.61 (s, 4H), 2.39 (t, J = 7.2 Hz, 4H), 1.55 (d, J = 9.8 Hz, 4H), 1.20 (s, 37H), 0.82 (t, J = 6.5 Hz, 6H). $^{13}$C NMR (400 MHz, DMSO-d6) δ ppm: 144.3, 141.0, 134.9, 126.3, 124.0, 36.6, 31.7, 29.5, 29.4, 29.2, 29.1, 29.0, 25.4, 22.5, 14.4. TOF-HRMS (m/z): Calcd. for (C$_{50}$H$_{74}$Br$_2$N$_4$O$_2$): 762.5812 (M – 2Br$^-$), found 761.5722 (M – 2Br$^-$/H$^+$).

**Synthesis of PyNp-C1**

This compound was prepared similarly to PyNp-C13 with a yield of 56%. $^1$H NMR (400 MHz, DMSO-$d_6$) δ ppm: 11.21 (s, 2H), 9.37 (s, 2H), 8.75 (d, J = 5.9 Hz, 2H), 8.50 (d, J = 8.4 Hz, 2H), 8.17 (dd, J = 8.6, 5.9 Hz, 2H), 8.10 (d, J = 8.2 Hz, 2H), 7.54 (t, J = 7.7 Hz, 2H), 6.89 (d, J = 7.3 Hz, 2H), 6.62 (s, 4H), 2.11 (s, 6H). $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ ppm: 170.4, 140.3, 135.7, 134.8, 134.8, 131.6, 130.6, 129.6, 129.5, 128.7, 126.6, 64.5, 24.3. TOF-HRMS (m/z): Calcd. for (C$_{26}$H$_{26}$Br$_2$N$_4$O$_2$): (M-2Br): 426.2045, found 425.1962 (M-2Br-H).

![Fig. S1 UV titration curve of UD at 409 nm versus equivalents of PyNp-C13.](image)
Fig. S2 (a) Fluorescence intensity of UD at 515 nm versus the number of equivalents of PyNp-C13. (b) The binding constant of PyNp-C13/UD using the Benesi-Hildebrand method.

Fig. S3 Lifetime of UD (6 µM) and PyNp-C13/UD (12 µM/6 µM) at 515 nm in a HEPES solution (10 mM, pH = 7.4).

Fig. S4 Tyndall experiments of UD (6 µM, a), PyNp-C13 (12 µM, b) and PyNp-C13/UD (12µM/6 µM) in HEPES (10 mM, pH = 7.4) buffer solution.
Fig. S5 (a) UV-vis spectra of PyNp-C13/UD (12µM/6 µM, λ<sub>ex</sub> = 490 nm) upon addition of various amounts of ATP in a HEPES (10 mM, pH = 7.4) buffered solution. The inset shows the color change of PyNp-C13/UD before and after addition of 8.0 equivalent of ATP in the day light. (b) UV titration curve of PyNp-C13/UD at 409 nm versus the equivalents of ATP.

Fig. S6 (a) Fluorescence intensity of PyNp-C13/UD at 515 nm versus the number of equivalents of ATP. (b) The binding constant of PyNp-C13/ATP as determined via the Benesi-Hildebrand method.
Fig. S7 The UV-vis (a) and fluorescence (c) spectra of PyNp-C13/UD (12 µM/6 µM, $\lambda_{ex} = 490$ nm) upon addition of various amounts of ADP in a HEPES (10 mM, pH = 7.4) buffered solution. (b) and (d) show the corresponding absorbance at 409 nm and the fluorescence at 515 nm versus the number of equivalents of ADP.

Fig. S8 UV-vis (a) and fluorescence (c) spectra of PyNp-C13/UD (12µM/6 µM, $\lambda_{ex} = 490$ nm) upon addition of various amounts of PP$_i$, in HEPES (10 mM, pH = 7.4) buffer solution. (b) and (d) show the corresponding absorbance at 409 nm and the fluorescence at 515 nm versus the number of equivalents of PP$_i$. 
Fig. S9 UV-vis (a) and fluorescence (c) spectra of PyNp-C13/UD (12 µM/6 µM, $\lambda_{ex} = 490$ nm) upon addition of various amounts of AMP in a HEPES (10 mM, pH = 7.4) buffered solution. (b) and (d) show the corresponding absorbance at 409 nm and the fluorescence at 515 nm versus the number of equivalents of AMP.

Fig. S10 size distribution of PyNp-C13/UD (12µM/6 µM) assembly before (a) and after the addition of 10 equivalents of ATP (b), ADP (c) and PPi (d) in aqueous solutions.
Fig. S11 (a) Fluorescence spectra of UD (6 µM, $\lambda_{ex} = 490$ nm) upon the addition of various amounts of PyNp-C1 in a HEPES (10 mM, pH = 7.4) buffered solution. The inset shows the fluorescence intensity of UD at 515 nm versus the equivalents of PyNp-C1. (b) The binding constant of PyNp-C1/UD as determined via Benesi-Hildebrand method.

Fig. S12 (a) Fluorescence spectra of PyNp-C1/UD (12µM/6 µM, $\lambda_{ex} = 490$ nm) upon the addition of various amounts of ATP in a HEPES (10 mM, pH = 7.4) buffered solution. The inset shows fluorescence intensity of PyNp-C1/UD at 515 nm versus the number of equivalents of ATP. (b) Binding constant of PyNp-C1/ATP as obtained via Benesi-Hildebrand method.
Fig. S13 Partial $^1$H NMR spectra of PyNp-C13 (4 mM), PyNp-C13/UD (8 mM/4 mM) and PyNp-C13/ATP (8 mM/4 mM) in DMSO-d$_6$/D$_2$O (8:2, v/v) solutions.

Fig. S14 Competitive fluorescence experiment of probe PyNp-C13/UD toward ATP in the presence of various anions in HEPES (10 mM, pH = 7.4) buffer solutions. 1-ADP, 2-PP$_i$, 3-AMP, 4-H$_2$PO$_4^-$, 5-AcO$^-$, 6-F$^-$, 7-Cl$^-$, 8-Br$^-$, 9-I$^-$, 10-CO$_3^{2-}$, 11-SO$_4^{2-}$.
**Fig. S15** Cell viability after incubation of Hepa1-6 murine liver cancer cells with various concentrations of PyNp-C13/UD in aqueous solutions.

**Fig. S16** Fluorescent monitoring of the ATP level in living *C. elegans*. A1 and A2: *C. elegans* alone; B1 and B2: pretreated with PyNp-C13/UD (40 μM /20 μM) for 3 h; C1 and C2: nematodes were first treated with 100 μM ATP for 4h and then incubated with PyNp-C13/UD (40 μM /20 μM). Scale bar: 250μm.
Fig. S17 $^1$H NMR spectrum of PyNp-C13 in a DMSO-$d_6$ solution

Fig. S18 $^{13}$C NMR spectrum of PyNp-C13 in a DMSO-$d_6$ solution
Fig. S19 ESI-TOF-MS spectrum of PyNp-C13

Fig. S20 $^1$H NMR spectrum of PyNp-C1 in a DMSO-$d_6$ solution
Fig. S21 $^{13}$C NMR spectrum of PyNp-C1 in a DMSO-$d_6$ solution

Fig. S22 ESI-TOF-MS spectrum of PyNp-C1
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<tr>
<th>Object</th>
<th>Binding constant</th>
<th>Detection limit (nM)</th>
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<tr>
<td>ATP</td>
<td>1.02×10^{11} M^{-2}^a</td>
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**Table S1** Summary of the binding constants and the detection limit of PyNp-C13 toward various anions in HEPES (1.0 mM, pH = 7.4) buffer solutions. ^a 2:1 binding mode, ^b 1:1 binding mode.

**References:**