

Taurine Boosts Cellular Uptake of Small D-peptides for Enzyme-Instructed Intracellular Molecular Self-assembly

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S1. Experimental materials and instruments

All the solvents and chemical reagents were used directly as received from the commercial sources without further purification. The hydrophilic products (**1a/b**, **4**) were purified with Water Delta600 HPLC system, equipped with an XTerra C18 RP column and an in-line diode array UV detector, hydrophobic product **3a/b** with flash chromatography. $^1\text{H-NMR}$ spectra were got on Varian Unity Inova 400, LC-MS spectra on a Waters Acuity ultra performance LC with Waters MICRO-MASS detector, rheological data on TA ARES G2 rheometer with 25 mm cone plate, TEM images on Morgagni 268 transmission electron microscope, confocal microscopy images on Leica TCS SP2 spectral confocal microscope or Marianas Spinning Disk confocal microscope. Esterase (from porcine liver, Sigma Product Number: E3019-20KU) was purchased from Sigma-Aldrich.

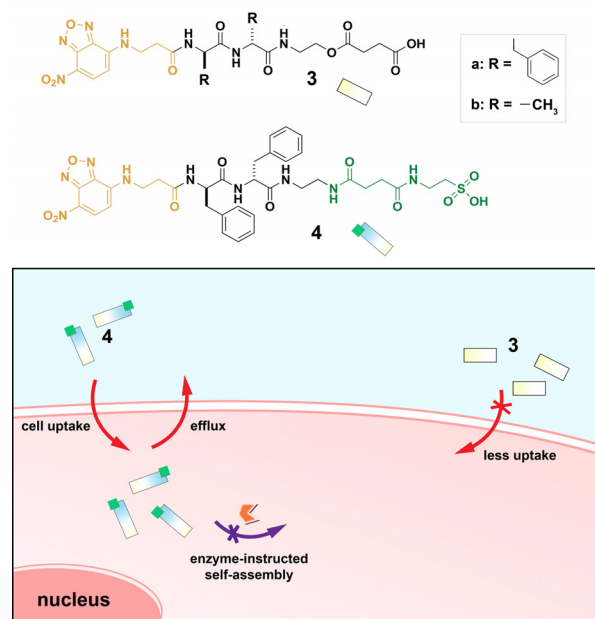
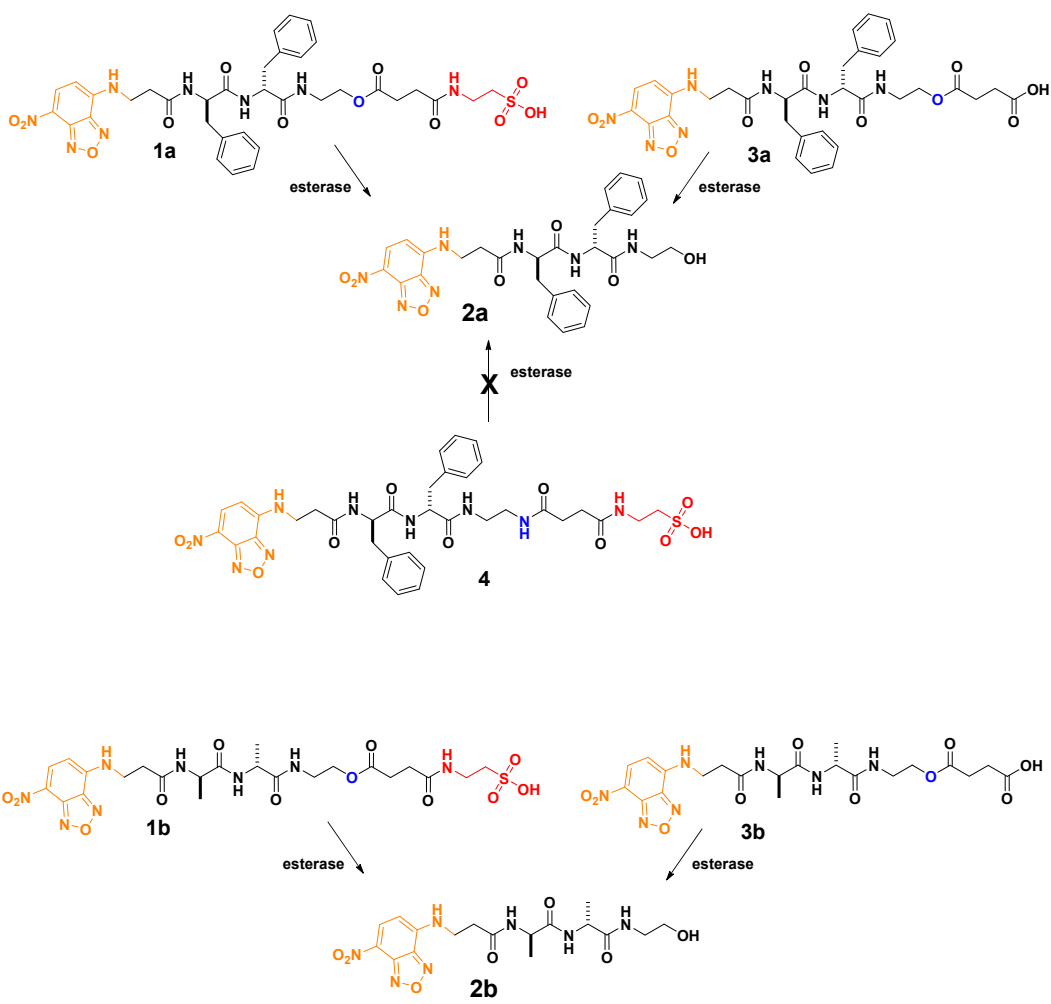


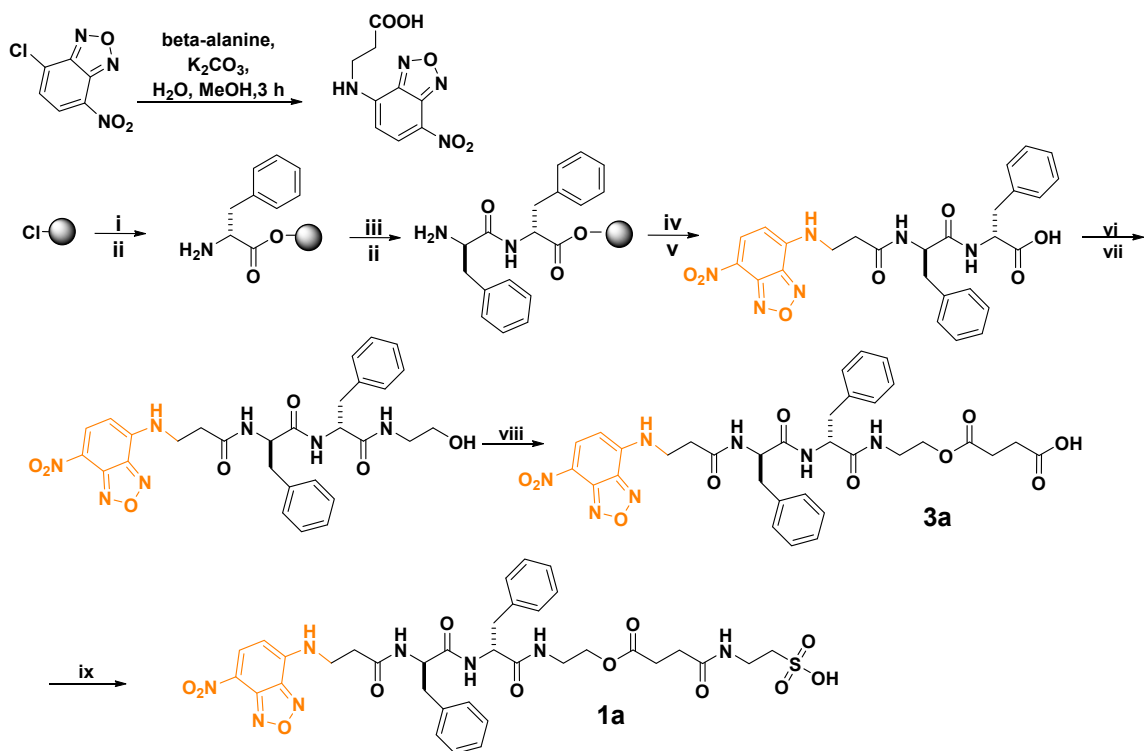
Figure S1. (A) Molecular structures of the relevant control molecules (**3a**, **3b** & **4**) and cellular uptake of these molecules.

S2. Synthesis and characterizations

We prepared the hydrogelator precursors (**1a/b**, **3a/b**) and the control (**4**) by combining solid phase^[1] and liquid phase peptide synthesis in fair yields (50-70%) and reasonable scales (0.1-0.5 g). The standard solid-phase peptide synthesis (SPPS)^[1] uses 2-chlorotriyl chloride resin (100-200 mesh and 0.3-0.8 mmol/g) and N-Fmoc-protected amino acids with side chains properly protected. Before that, we prepared NBD-COOH, which was directly used in SPPS, from NBD-Cl based on literature^[2]. The following scheme (Figure S1) illustrates the synthetic procedure of **1a/3a**, and the synthesis of **4**. The synthetic route of **1b/3b** is the same with that of **1a** and **3a**.

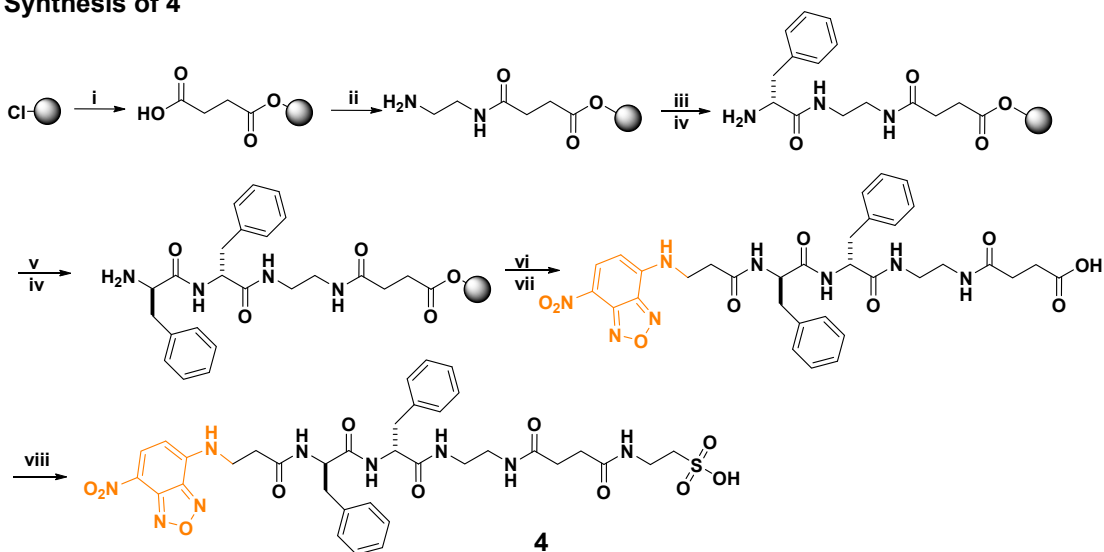


Synthesis of 1a and 3a



i) Fmoc-D-Phe-OH, DIEA; ii) 20 % piperidine; iii) Fmoc-D-Phe-OH, HBTU, DIEA; iv) NBD-COOH, HBTU, DIEA; v) 90 % TFA in water; vi) NHS, DIC; vii) ethanolamine; viii) DIEA, succinic anhydride; ix) HBTU, DIEA, taurine

Synthesis of 4



i) succinic acid, DIEA; ii) ethylenediamine, DIEA; iii) Fmoc-D-Phe-OH, HBTU, DIEA; iv) 20% piperidine; v) Fmoc-D-Phe-OH, HBTU, DIEA; vi) NBD-COOH, HBTU, DIEA; vii) 90 % TFA in water; viii) HBTU, DIEA, taurine

Figure S2. The chemical structure of 1a/b, 2a/b, 3a/b, 4, and the synthetic route of 1a, 3a and 4

1a: $^1\text{H-NMR}$ (400 MHz, $\text{DMSO-}d_6$) δ 9.27 (s, 1H), 8.20-8.11 (m, 3H), 7.94 (d, $J = 8.0$ Hz, 1H), 7.77 (t, $J = 4.0$ Hz, 1H), 7.68 (d, $J = 4.0$ Hz, 1H), 7.50 (t, $J = 8.0$ Hz, 1H), 7.37 (t, $J = 8.0$ Hz, 1H), 7.21 - 7.05 (m), 4.44 (m, 1H), 3.90 (t, $J = 4.0$ Hz, 1H), 3.61 - 3.50 (m, 4H), 3.27 - 3.17 (m, 2H), 3.10 - 3.07 (m, 4H), 2.92 - 2.85 (m, 1H), 2.81 - 2.72 (m, 1H), 2.64 (t, $J = 12.0$ Hz, 1H), 2.57 (t, $J = 8.0$ Hz, 1H), 2.46 - 2.43 (m, 3H), 2.29 (t, $J = 8.0$ Hz, 1H). ESI MS(m/z) [M] $^+$ calcd. for $\text{C}_{35}\text{H}_{40}\text{N}_8\text{O}_{12}\text{S}$, 797.3146 found [M-H] $^-$ 795.2994.

3a: $^1\text{H-NMR}$ (400 MHz, $\text{DMSO-}d_6$) δ 12.21 (s, 1H), 8.28 (d, $J = 8.0$ Hz, 1H), 8.18 (d, $J = 8.0$ Hz, 1H), 8.05 (t, $J = 4.0$ Hz, 1H), 7.85 (d, $J = 8.0$ Hz, 1H), 7.78 (d, $J = 8.0$ Hz, 1H), 7.75 (d, $J = 8.0$ Hz, 1H), 7.60 (s, 1H), 7.47 (m, 2H), 7.16 - 7.2 (m, 5-6H), 4.54 (td, $J = 8.0, 4.0$ Hz, 1H), 4.47 (td, $J = 8.0, 4.0$ Hz, 1H), 3.94 (t, $J = 8.0$ Hz, 2H), 3.54 (q, $J = 12.0$ Hz, 2H), 2.93 - 2.99 (m, 2H), 2.85 - 2.71 (m, 2H), ESI MS(m/z) [M] $^+$ calcd. for $\text{C}_{33}\text{H}_{35}\text{N}_7\text{O}_{10}$, 690.2727 found [M-H] $^-$ 688.3225.

4: $^1\text{H-NMR}$ (400 MHz, $\text{DMSO-}d_6$) δ 9.28 (s, 1H), 8.30-8.05 (m, 3H), 7.94 (d, $J = 8.0$ Hz, 1H), 7.87 (t, $J = 4.0$ Hz, 1H), 7.64 (d, $J = 16.0$ Hz, 1H), 7.48 (t, 1H), 7.39 (t, $J = 8.0$ Hz, 1H), 7.11 - 7.90 (m), 4.37-4.52 (m, 1H), 3.56 (m, 4H), 3.09 (m, 4H), 2.92 - 2.85 (m, 1H), 2.81 - 2.72 (m, 1H), 2.64 (t, $J = 12.0$ Hz, 1H), 2.57 (t, $J = 8.0$ Hz, 1H), 2.46 - 2.43 (m, 3H), 2.29 (t, $J = 8.0$ Hz, 1H). ESI MS(m/z) [M] $^+$ calcd. for $\text{C}_{35}\text{H}_{41}\text{N}_9\text{O}_{11}\text{S}$, 796.2745 found [M-H] $^-$ 794.3245.

S3. TEM sample preparation.

In this paper, we used negative staining technique to study the TEM images. We first glow discharged the 400 mesh copper grids coated with continuous thick carbon film (~ 35 nm) prior to use to increase the hydrophilicity. After loading samples (4 μL) on the grid, we then rinsed grid by dd-water for twice or three times. Immediately after rinsing, we stained the grid containing sample with 2.0 % w/v uranyl acetate for three times. Afterwards, we allowed the grid to dry in air.

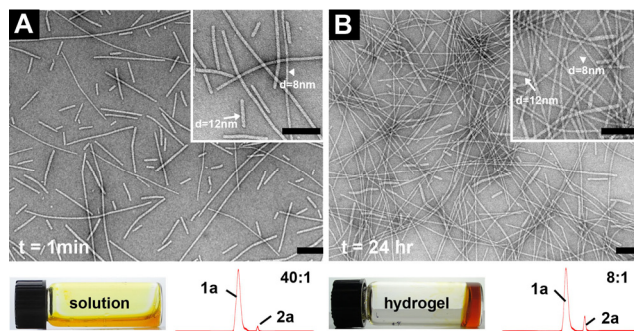


Figure S3. TEM images, optical images and analytical HPLC spectra of the solution or hydrogel formed by **1a** (1.0 wt%) after the addition of esterase (1 U/ml) for (A) 1 min; (B) 24 hours.

S4. General procedures for hydrogel preparation

Enzymatic gelation: We dissolved **1a/3a** into distilled water, and adjusted pH of the solution, monitored by pH paper, carefully by adding 1M NaOH. After the pH of the solution reached 7.4, we then added extra distilled water to make the final concentration, followed by the addition of esterase.

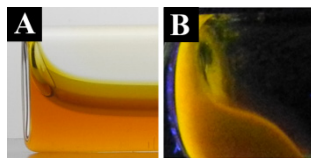


Figure S4. The optical image of (A) the solution of **3a** (1.0 wt %) and (B) hydrogel formed by treating the solution of **3a** with esterase (1 U/ml) under the excitation of a hand-held UV lamp ($\lambda_{\text{ex}} = 365 \text{ nm}$).

S5. Cell culture

The HeLa cell line (ATCC[®] CCL-2[™]) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The HeLa cells were propagated in Minimum Essential Media (MEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics in a fully humidified incubator containing 5% CO₂ at 37°C. The dynamin 1, 2, and 3 TKO mouse fibroblast (TKO) cells were provided by Prof. De Camilli Lab at Yale University and were propagated in DMEM+10% FBS + l-glutamine + 1% pen/strep in a fully humidified incubator containing 5% CO₂ at 37°C.

S6. Sample preparation for confocal microscopy

HeLa cells in exponential growth phase were seeded in glass bottomed culture chamber at 1×10^5 cell/well. The cells were allowed for attachment for 12 h at 37 °C, 5% CO₂. The culture medium was removed, and new culture medium containing **1a/b**, **3a/b** or **4** at 200 μM was added. After incubation for certain time, cells were stained with 1.0 $\mu\text{g}/\text{ml}$ Hoechst 33342 for 30 min at 37 °C in dark. After that, cells were rinsed three times by PBS buffer, and then kept in the live cell imaging solution (Invitrogen Life Technologies A14291DJ) for imaging.

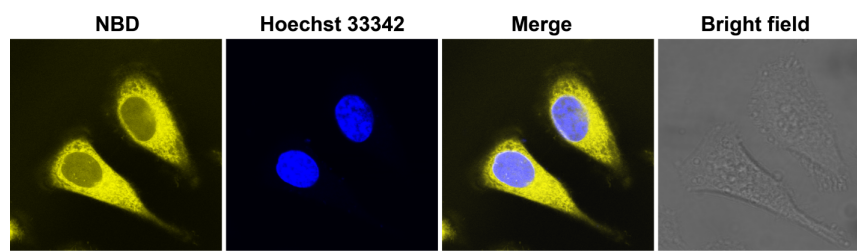


Figure S5. High resolution confocal images of HeLa cells incubated with **1a** (200 μM) for 24h. The scale bar = 10 μm .

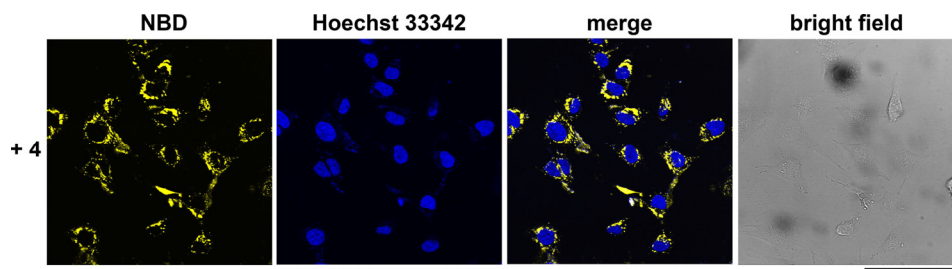


Figure S6. Fluorescent confocal microscopy images show the fluorescence emission in HeLa cells with the treatment of **4** at the concentration of 200 μM in culture medium for 24 h and stained with Hoechst 33342 (nuclei). The scale bar is 50 μm .

S7. Procedure of fluorescence staining of F-actin filaments

1. Allow cells attaching on the glass-bottom confocal petri dish (3cm) for normal culture in medium or treated with certain compound (1, 500 μM) for a specific time (24 hr);
2. Wash the cells with 1 \times PBS twice;
3. Add 2 mL of freshly prepared 4% paraformaldehyde to each petri dish for 30 min incubation at room temperature;
4. Wash the cells with 1 \times PBS twice;
5. Add 1 mL of 0.1% Triton X-100 in PBS buffer to each petri dish and incubate 30 min to make the cells permeable;
6. Wash the cells with 1 \times PBS twice;
7. Add 1 mL of 0.1% BSA in PBS buffer to each petri dish and incubate 30 min to block non-specific following dyes binding sites;
8. Wash the cells with 1 \times PBS twice;
9. Add 1 mL of PBS buffer containing 5 units of Alexa Fluor® 633 phalloidin to each petri dish and incubate 60 min for F-actin staining;
10. Wash the cells with 1 \times PBS twice;
11. Add 1 mL of 1 $\mu\text{g}/\text{mL}$ of Hoechst 33342 to each petri dish and incubate 10 min for nuclei staining;
12. Wash the cells with 1 \times PBS twice;
13. Mount the petri dish for fluorescence imaging.

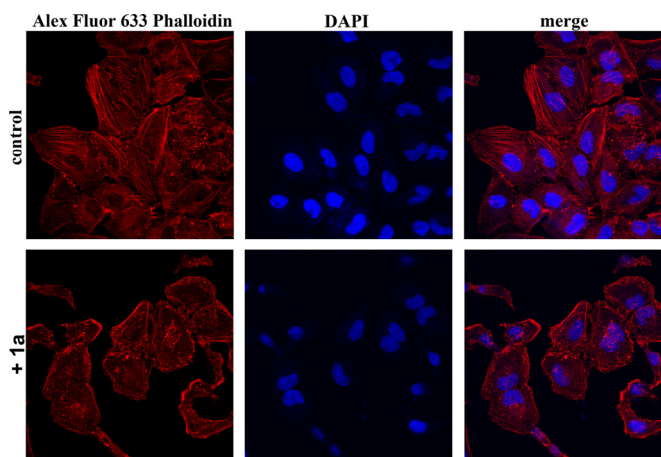


Figure S7. Fluorescent confocal microscopy images show the HeLa cells (upper) without or (bottom) with the treatment of **1a** (500 μM) in culture medium and stained with Hoechst 33342 (nuclei) and Alex Fluor 633 Phalloidin (F-actin). The scale bar is 50 μm .

S8. Cellular uptake measurement

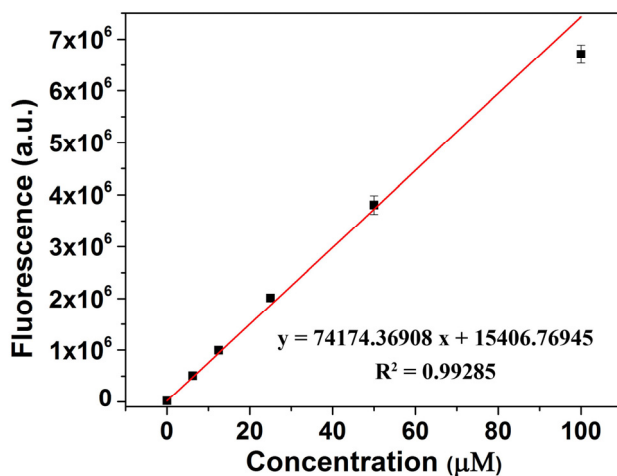
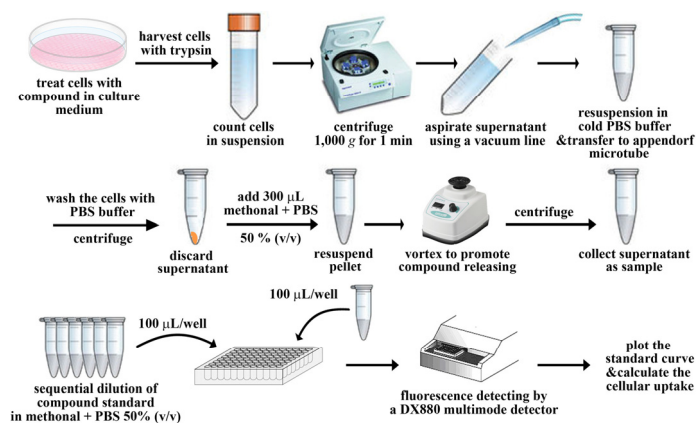


Figure S8. (A) Schematic illustration of the procedure of cellular uptake measurement. (B) Standard curve

*Intracellular concentration = $(c \times 300 \mu\text{L}) / (\text{cell number} \times 4 \times 10^{-9} \text{ cm}^3)$

$c = (\text{fluorescence} - 15406.76945) / 74174.36908$

*An average size for the common cells used in cell culture (HeLa) is 15-20 microns in diameter for a suspended cell (volume $4/3\pi r^3 = 4000 \mu\text{m}^3$ or $4 \times 10^{-9} \text{ cm}^3$).^[3]

*The standard curves of **1a/b**, **2a/b**, **3a/b**, and **4** are almost identical, so we take their average.

*We use methanol to break cell membrane and help the **1a**, **4**, and **2a** fully dissolved.

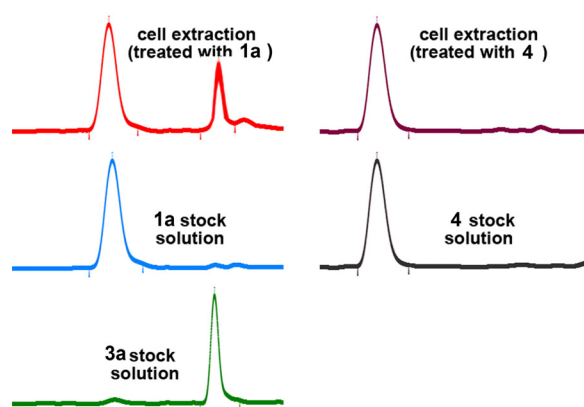


Figure S9. Cell extraction analysis by analytical HPLC, detected at $\lambda = 480 \text{ nm}$. The spectra shown above are all in the same time scale.

S9. Light scattering sample preparation

The static light scattering experiments were performed using an ALV (Langen, Germany) goniometer and correlator system with a 22 mW HeNe ($\lambda = 633 \text{ nm}$) laser and an avalanche photodiode detector. All samples were filtered by using $0.22 \mu\text{m}$ filters after heating. The addition of ALP to the solution of precursors for 24 h, we obtained corresponding samples of hydrogelators. The SLS tests were carried out at room temperature, and the angles of light scattering we chose were 30° , 60° , 90° , and 120° , respectively. The resulting intensity ratios are proportional to the amount of aggregates in the samples.

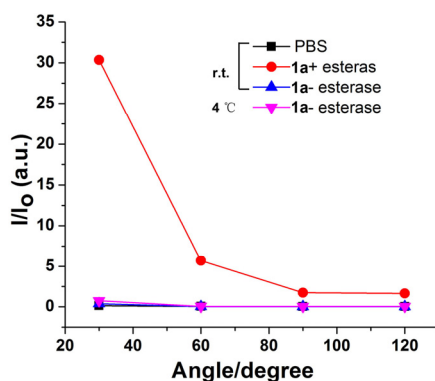


Figure S10. Static light scattering (SLS) signals of the solution of **1a** (100 μM) in PBS buffer without and with the addition of esterase at different temperatures.

S10. Endocytosis mechanism elucidation (Endocytosis Quantification)

TKO Cell culture:

The deletion of dynamin in these cells is mediated by a tamoxifen inducible KO strategy.^[4] Briefly, these cells express a Cre-estrogen receptor mutant knock-in transgene from the ROSA26 locus. Thus, Cre is only shuttled into the nucleus in response to tamoxifen (OHT) exposure. Dynamin disappears within the first 3-4 days after initiating OHT treatment but that the last little bit makes a big difference as the full phenotype takes another 5-6 days to appear. Experiment is carried out on day 5 or 6.

1. Seed cell at initial concentration of 5,000-10,000 cells/confocal dish to ensure sub-confluency.
2. 12 hours later, treat cells with 4-hydroxytamoxifen (OHT) (3 μM) in complete culture medium (DMEM+10% FBS + L-glutamine + pen/strep), and incubate for 48 hours.
3. Change medium to normal growth medium with low concentration of OHT (300 nM) and incubate for another 3-4 days. Dynamin TKO cells do not proliferate at this point.
4. On the fifth or sixth day, do experiment.
5. Control cells are the same cells without the treatment of OHT.
6. In addition, treat control cells with different inhibitors to determine the pathways of the cell entry of **1a**.

Compound treatment:

1. Treat TKO or control cells with NBDffestau (500 μM) in complete growth medium and incubate for 1 hour.
2. Or Pretreat control cells with endocytosis inhibitors (30 μM chlorpromazine; 5 $\mu\text{g/mL}$ filipin III; 100 μM ethyl-isopropyl-amiloride (EIPA)) for 0.5 hr and then treat the cells with both **1a** (500 μM) and inhibitors and incubate for 1 hour.

Imaging:

1. For each condition, pick 8-10 cells for quantifications and calculate standard deviations.

2. Wash cells with Live Cell Imaging Solution for 3 times, and then add 1-2 ml of imaging solution for imaging.
3. Fluorescence images are taken on Marianas Spinning disk confocal microscope with 63X oil object.
4. All the fluorescent images and Z scan are taken with an exposure of 500 ms (488 nm).
5. Fluorescent spots in control (with and without inhibitors) and TKO cells are analyzed using imageJ.

Images processing and quantification:

1. A 4 μm thick section was imaged from the bottom of the cell at 0.27 μm intervals in the z-axis with exposure time of 500 ms
2. The slices were collapsed or binned to a single image by maximal intensity Z-projection with imageJ.
3. Substrate background with rolling ball radius of 50 pixels. (*process-subtract background*)
4. Activate thresholding (*image-adjust-threshold*). Pick pixels between 20 -255 greyscale.
5. Analyze particle (*analyze-analyze particles*) (size (pixel²): 5-infinity; circularity 0.00-1.00; show outline; display results and summarize.
6. Get distribution based on puncta areas
7. Calculate average fluorescent puncta density and standard deviation.

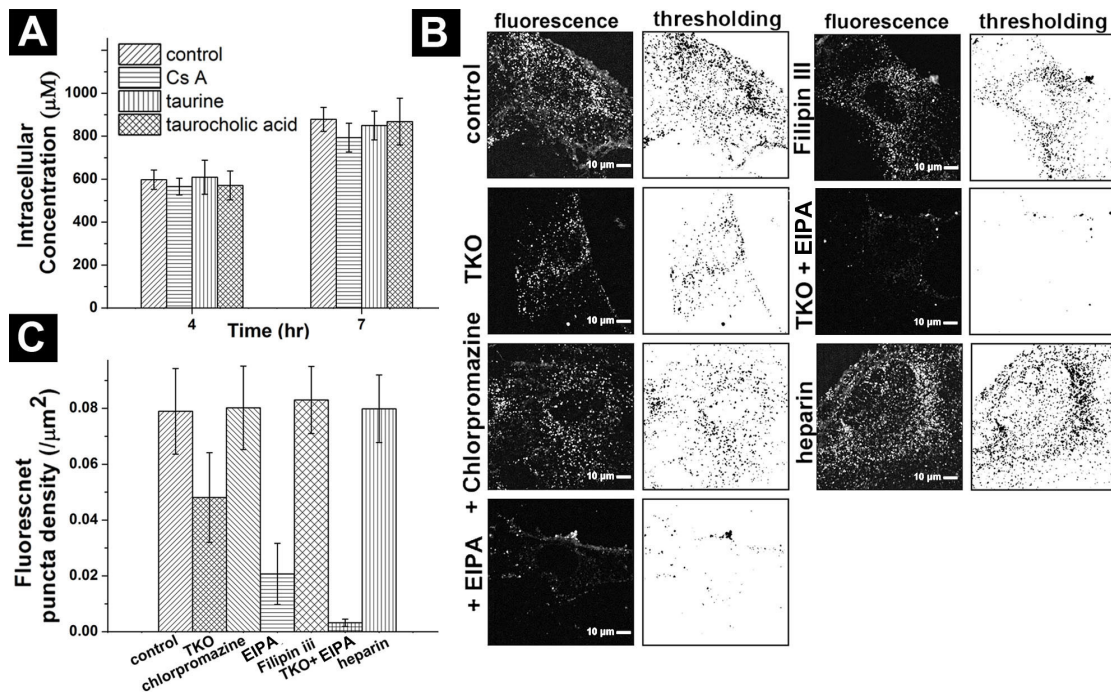


Figure S11. (A) C_u of 1a inside HeLa cells after the addition of Cs A (5 μM), taurine (3 mM), taurocholic acid (1 mM). The incubating concentration of 1a is 200 μM . (B) Gray scale confocal microscopy images show impaired cellular uptake of 1a in TKO cells and normal cells treated with EIPA. (C) Quantification of cellular uptake of 1a at different conditions in (B). Quantification procedure is shown in SI.

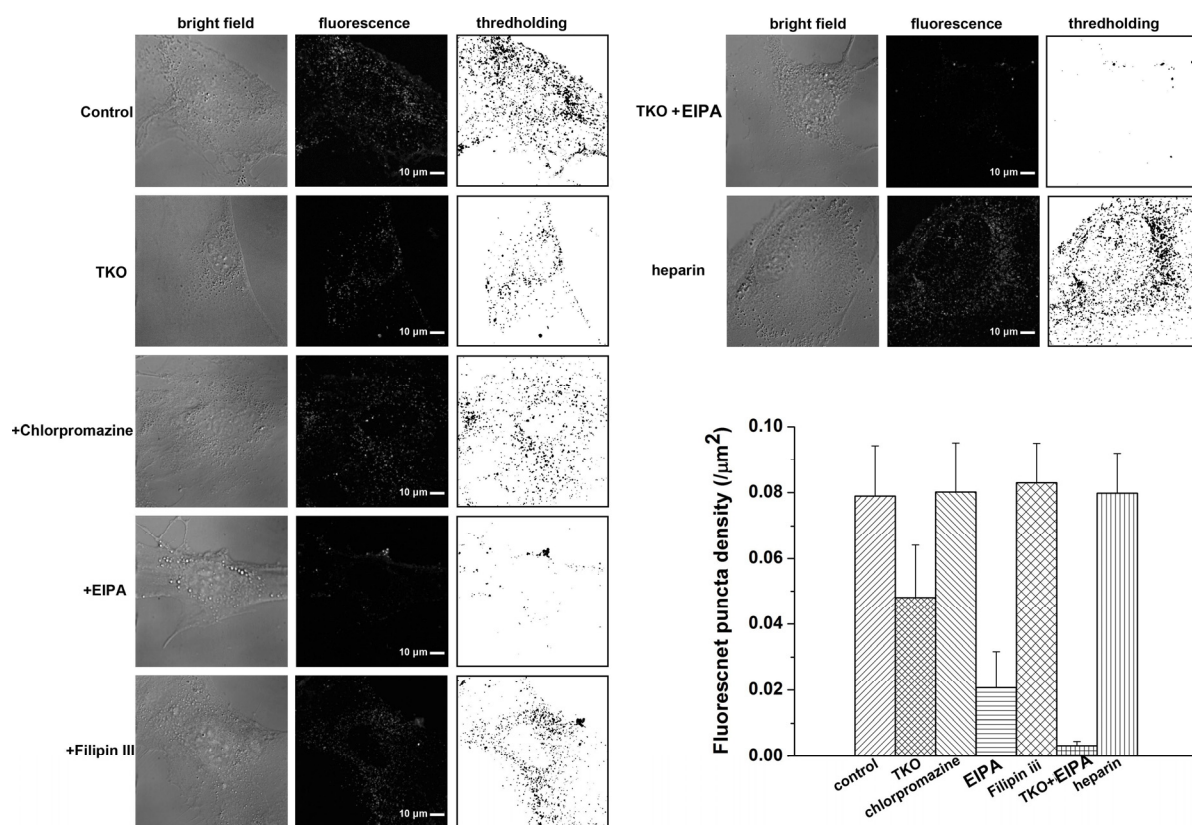


Figure S12. Impaired internalization of fluorescent **1a** in TKO cells and normal cells treated with EIPA. These fluorescent puncta are known to reflect the accumulation of **1a** in cells. Quantifications of the fluorescence signals for all conditions are shown on the right (8-9 cells per condition). Scale bars: 10 μm .

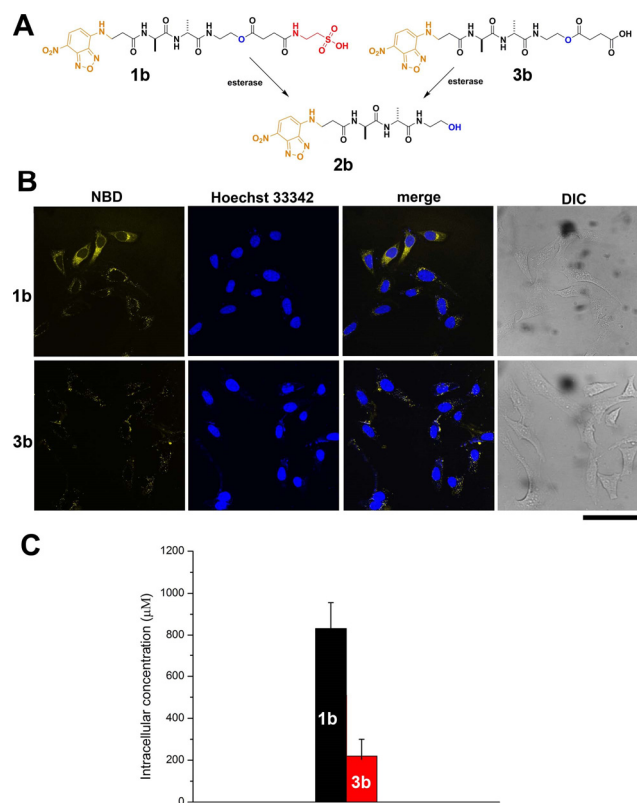


Figure S13. (A) Chemical structures of **1b** and **3b**, and their hydrolysis by esterase to form **2b**. (B) Fluorescent confocal microscopy images show the fluorescence emission in HeLa cells with the treatment of **1b** and **3b** at the concentration of 200 µM in culture medium for 24 h and stained with Hoechst 33342 (nuclei). The scale bar is 50 µm. (C) Intracellular concentration C_u of **1b** and **3b** after 24-hour incubation with these two molecules (200 µM).

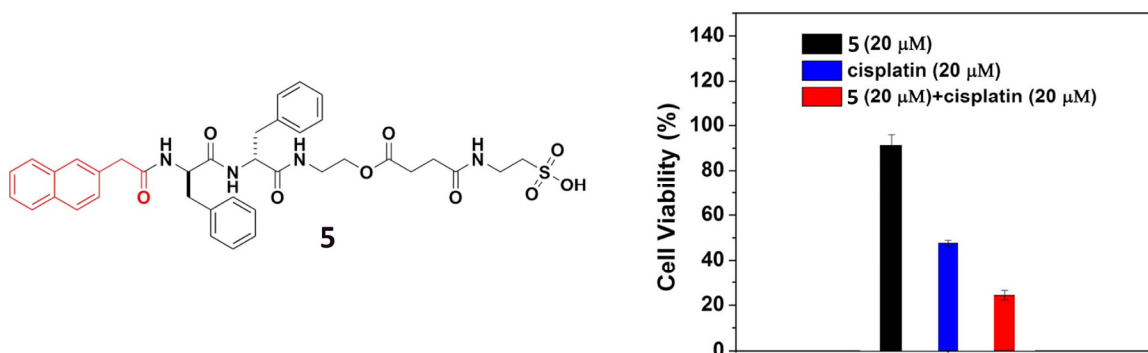


Figure S14. Relative cell viability of SK-OV-3 cells incubated with 20 µM **5**, 20 µM cisplatin, or the mixture of 20 µM **5** and 20 µM cisplatin. The initial number of cells is 1.0×10^4 /well.

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