Supporting Information for:

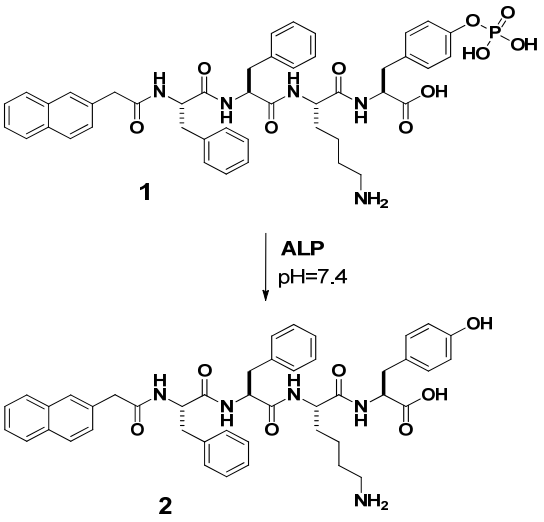
Imaging Self-assembly Dependent Spatial Distribution of Small Molecules in Cellular Environment

Yuan Gao, Yi Kuang, Xuewen Du, Jie Zhou, Preethi Chandran, Ferenc Horkay and Bing Xu*

Contents

S1. The molecular structure of **2**

Scheme S1. The molecular structure of **2**, which is the product of the dephosphorylation of **1**.



S2. Critical gelation concentration (CGC) of **3a**

Figure S1. After the addition of 20 U/mL of ALP to a solution of **2a** at the concentration of (A) 2 mM, (B) 1 mM, and (C) 500 μM, only (A) forms a self-supported hydrogel while (B) is a highly viscous solution and (C) becomes turbid with lots of smaller aggregates. Thus, the CGC of **3a** is between 1 and 2 mM (0.2 wt%).

S3. Self-assembly behavior of **2c**

S3.1. TEM and optical images of the solution of **2c**

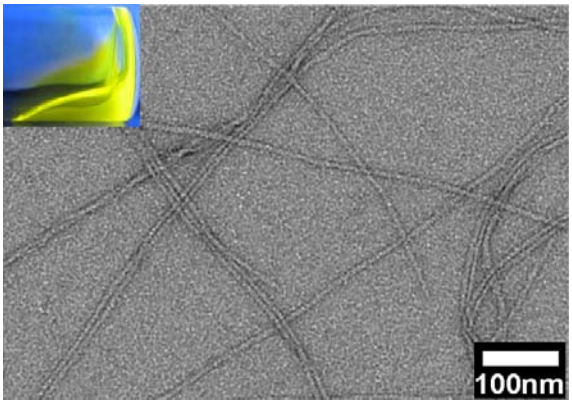


Figure S2. TEM image reveals the existence of nanofibers in a viscous solution of **2c** (0.6 wt%). (inset: excited by a hand-held UV lamp, the solution of **2c** gives a fluorescent emission)

S3.2. The use of HPLC to determine the enzymatic dephosphorylation profile of **2c** in aggregation state.

After adding the ALP into the viscous solution of **2c** (0.6 wt% in concentration), we find, at room temperature, 4.8% of **2c** being converted to **3c** (calculated by UV absorbance at 430 nm, a characteristic UV absorbance peak of DBD residue) in the first hour. The total conversion is only 7.0% three hours after adding ALP, indicating that the conversion increases little over time due to the self-assembly of **2c**. Then, we put the sample in a 37 ^{o}C water bath. The total conversion accumulates to 9.8% with the first 30 min at 37 ^{o}C, but only reaches to 11.8% after four more hours at 37 ^{o}C. Stirring of the reaction mixture, however, further increases the conversion up to 19.7% after one hour of stir. The final yield of **3c** is about 30% after 24 hours. The incomplete conversion of **2c** results a mixture of **2c** and **3c**, which fails to form a stable gel. Despite that there is a "burst" conversion of **2c** to **3c** after changing conditions (e.g., raising temperature or starting stirring) each time, the conversion quickly slows down. Since changing the conditions disfavor the self-assembly of **2c**, these results confirm that the nanofibers of **2c** or the nanofibers of the mixture of **2c** and **3c**, indeed, decreases the conversion of **2c** to **3c** catalyzed by ALP.

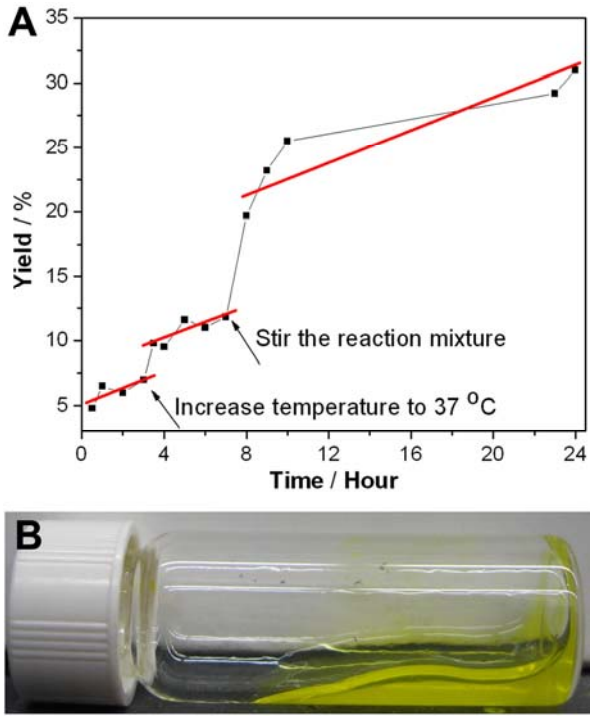


Figure S3. (A) The accumulated molar percentage of conversion from **2c** to **3c** at each moment when the condition changes are calculated according to analytical RP-HPLC trace. The start point is the addition of 20 U/mL of ALP into a viscous solution of **2c** (0.6 wt%). (B) After 24 hours, only 30% of **2c** get dephosphorylated to **3c** and the resulting reaction mixture of **2c** and **3c** remains a viscous solution instead of a self-supported hydrogel.

S4. Characterization of **2d**

S4.1. The molecular structure of **2d**

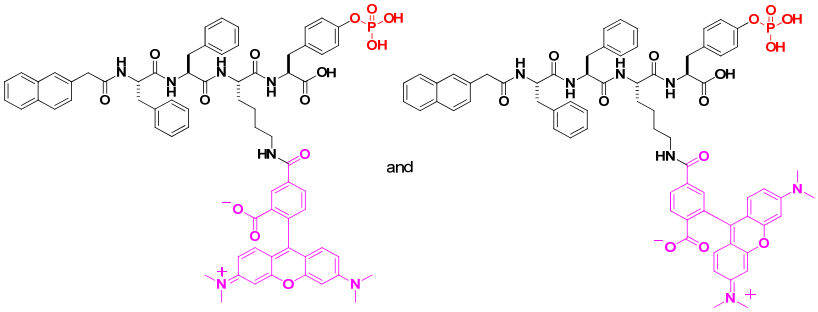


Figure S4. The molecular structure of **2d**. The commercial NHS-Rhodamine (Product No. 46406 from Thermo Scientific) is a mixture of 5-(and 6)-carboxytetramethylrhodamine, succinimidyl ester, which was used without the separation of the two forms of **2d**.

S4.2. Non-hydrogelator **3d**

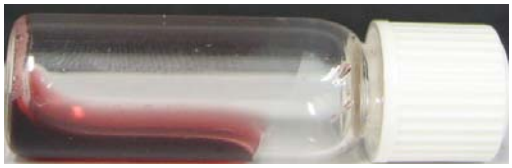


Figure S5. After the addition of 20 U/mL of ALP into the solution of **2d** (0.6 wt%, pH 7.4), optical image shows that the resulting solution of **3d** fails to form a hydrogel (or even a viscous solution).

S4.3. The fluorescence spectra of **2d** and **3d**

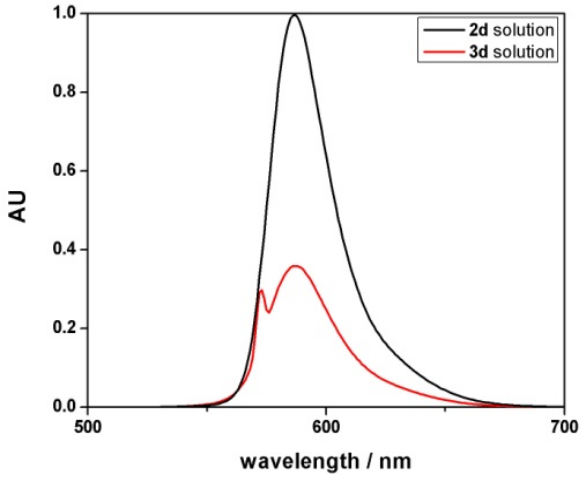


Figure S6. The fluorescence emission spectra of the solution of **2d** (0.012 wt%, pH 7.4) and the resulting solution of **3d** after dephosphorylation. (λ_{ex} = 573 nm) *Due to the high quantum yield of rhodamine, we can only obtain the emission spectrum of **2d** at the diluted concentration (94.9 μM).

S5. Dynamic light scattering: autocorrelation functions [(g(t)] of solutions of **1** before and after dephosphorylation

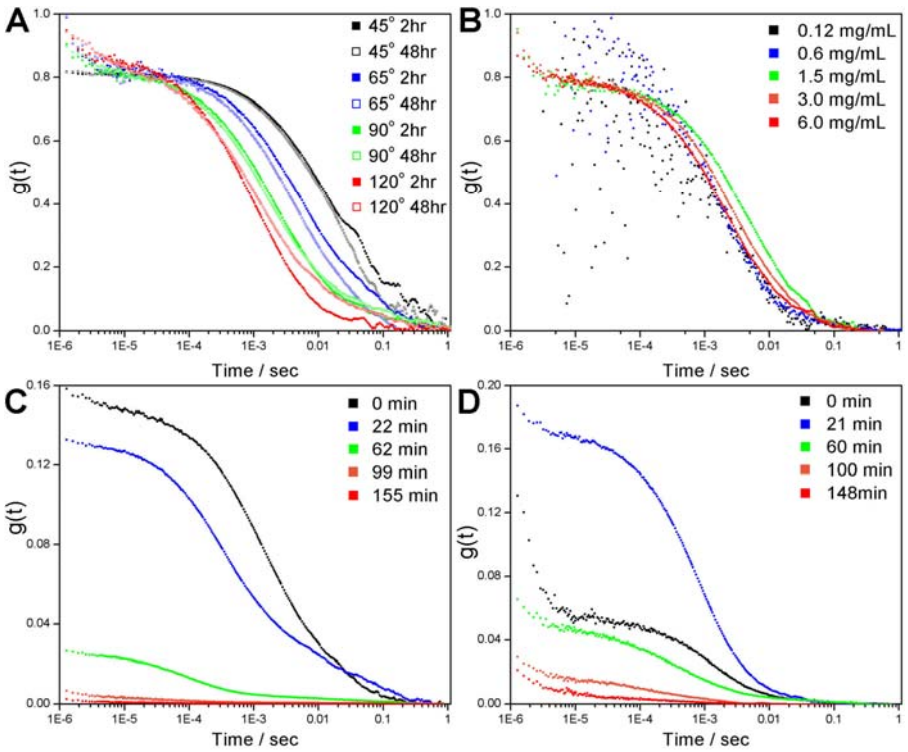


Figure S7. Dynamic light scattering of solutions of **1** at pH = 7 in water. (A) Angular dependence of the correlation functions in the 6 mg/mL solution of **1** after two hours and 48 hours. The autocorrelation decay curves remain almost the same after aging for 48 hours, which means the aggregates are stable in solution; (B) DLS decay curves of a serial dilution of **1** from 6 mg/mL to 0.12 mg/mL gradually become noisy, which reveals that the critical concentration for formation of supramolecular aggregates is between 0.6 mg/mL and 0.12 mg/mL; (C) DLS decay curves depict the kinetics of the formation of more aggregates in the solution of 6 mg/mL of **1** after addition of 10 U/mL of ALP; (D) Time dependent light scattering decay curves depict the kinetics of aggregate formation in the solution of 0.6 mg/mL of **1** after addition of 10 U/mL of ALP. All the measurements are made at $\theta=90^{o}$ if not otherwise indicated.

S6. The ITC experiment

Each precursor solution is prepared by dissolving the compound in PBS buffer to yield the solution at a suitable concentration. Titration experiments are performed in a Nano ITC (Isothermal Titration Calorimeter, 190 μL) with a gold reaction vessel. The reference cell was filled with water. Each titration experiment consists of 25 × 2 μL injections of precursors into PBS buffer at 600-second intervals with a stirring speed of 300 rpm. A 300-second baseline was collected before the first injection and after the last injection. Prior to starting the titration experiment, the calorimeter was equilibrated to a baseline with a drift of less than 100 nW over a ten-minute period (software design).

S7. Temporal profile of molecular assemblies

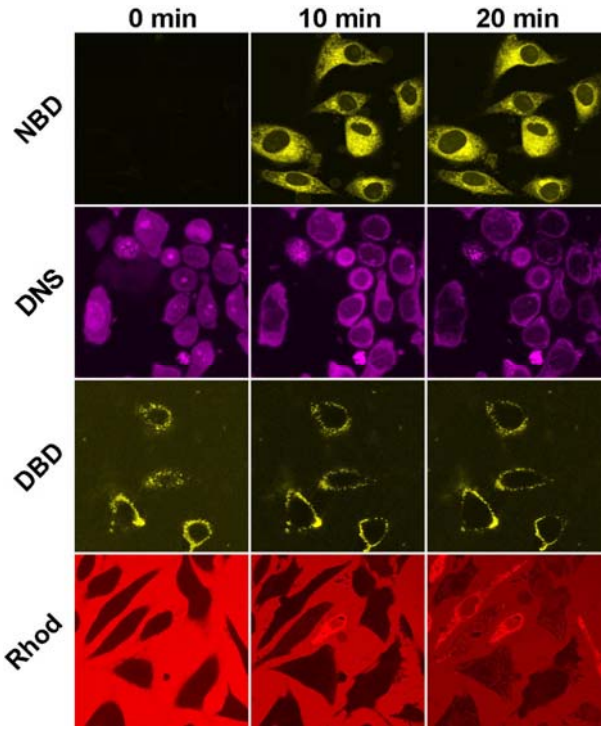


Figure S8. Fluorescent confocal images record the profile of the molecular self-assembly at three typical time points before 30 min. Each group of HeLa cells are incubated with 500 μM of **2a-2d** in PBS buffer for 30 min. (each frame: 125 μm x 125 μm)

S8. Spatiotemporal profile below CAC

S8.1. HeLa cells incubated with 200 nM of **2b**

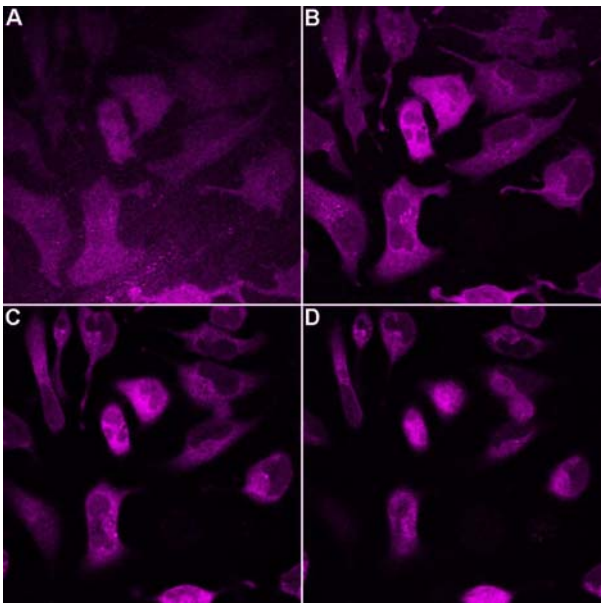


Figure S9. Four typical confocal fluorescent images from the Z-slices of a group of HeLa cells incubated with 200 nM of **2b** shows the homogeneous distribution of **3b** in the cytoplasm. (each frame: 150 μm x 150 μm; z step: 1.37μm)

S8.2. HeLa cells incubated with 10 μM of **2c**

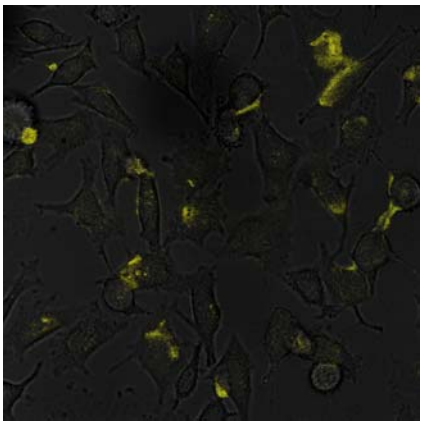


Figure S10. The merged fluorescent and bright field images of a group of HeLa cells incubated with 10 μM of **2c** shows that **2c** can penetrate the cell membrane. (each frame: 238 μm × 238 μm)

S9. The procedure of chemical fixation and fluorescence staining.

Procedure of the chemical fixation and fluorescence staining of F-actin filaments and nuclei:

1. Allow cells attaching on the glass-bottom petri dish (3cm) for normal culture in medium or treated with certain compound for a specific time;
2. Wash the cells with 1× PBS twice;
3. Add 1 mL of freshly prepared 4% paraformaldehyde to each petri dish and wait 45 min incubation at room temperature to fix the cells;
4. Wash the cells with 1× PBS twice;
5. Add 1 mL of 0.1% Triton X-100 in PBS buffer to each petri dish and wait 30 min to make the cells permeable;
6. Wash the cells with 1× PBS twice;
7. Add 1 mL of 0.1% BSA in PBS buffer to each petri dish and wait 30 min to block non-specific following dyes binding sites;
8. Wash the cells with 1× PBS twice;
9. Add 1 mL of PBS buffer containing 3 units of Alexa Fluor® 633 phalloidin to each petri dish and wait 60 min for F-actin staining;
10. Wash the cells with 1× PBS twice;
11. Add 1 mL of 2 μg/mL of DAPI to each petri dish and wait 10 min for nuclei staining;
12. Wash the cells with 1× PBS twice;
13. Mount the petri dish for fluorescence imaging.

S10. Latrunculin A (**4**) treatment

S10.1.1. HeLa cells (wild type) change morphology (movie)

The movie shows that a group of wild type HeLa cells change their shapes during the treatment of **4** at the concentration of 10 μM for 30 min while several individual cells show resistance to **4** without shape change.

S10.1.2. HeLa cells (wild type) visualized with F-actin / nuclei staining

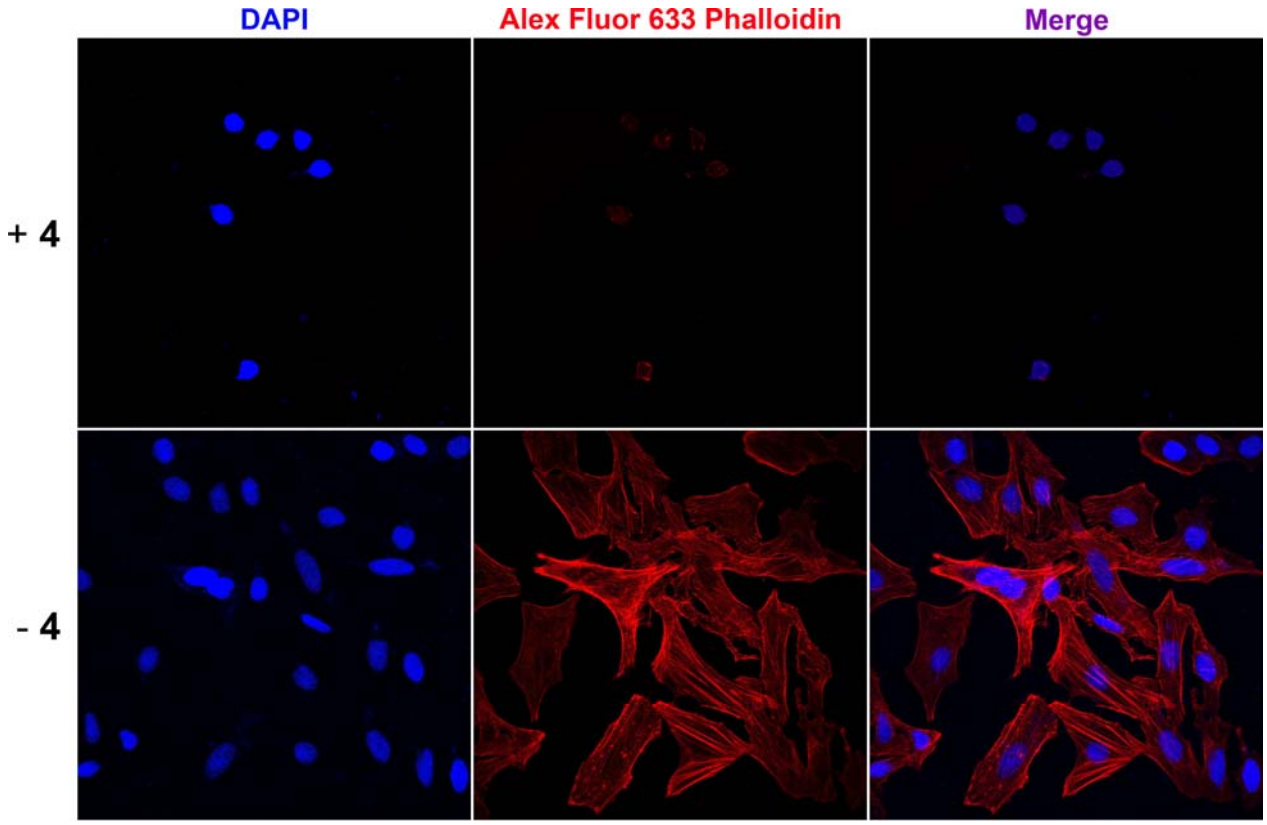


Figure S11. The fluorescence images show the HeLa cells (upper) after treatment of **4** or (bottom) without treatment of **4** and stained with DAPI (nuclei) and Alex Fluor 633 Phalloidin (F-actin). After treatment of **4**, most of cells detach and disappear due to the loss of F-actin. (Condition of treatment of **4**: 10 μM, 30 min. Each frame: 300 μm × 300 μm)

S10.2. HeLa cells (pre-treated with compounds **2a**) visualized with F-actin / nuclei staining

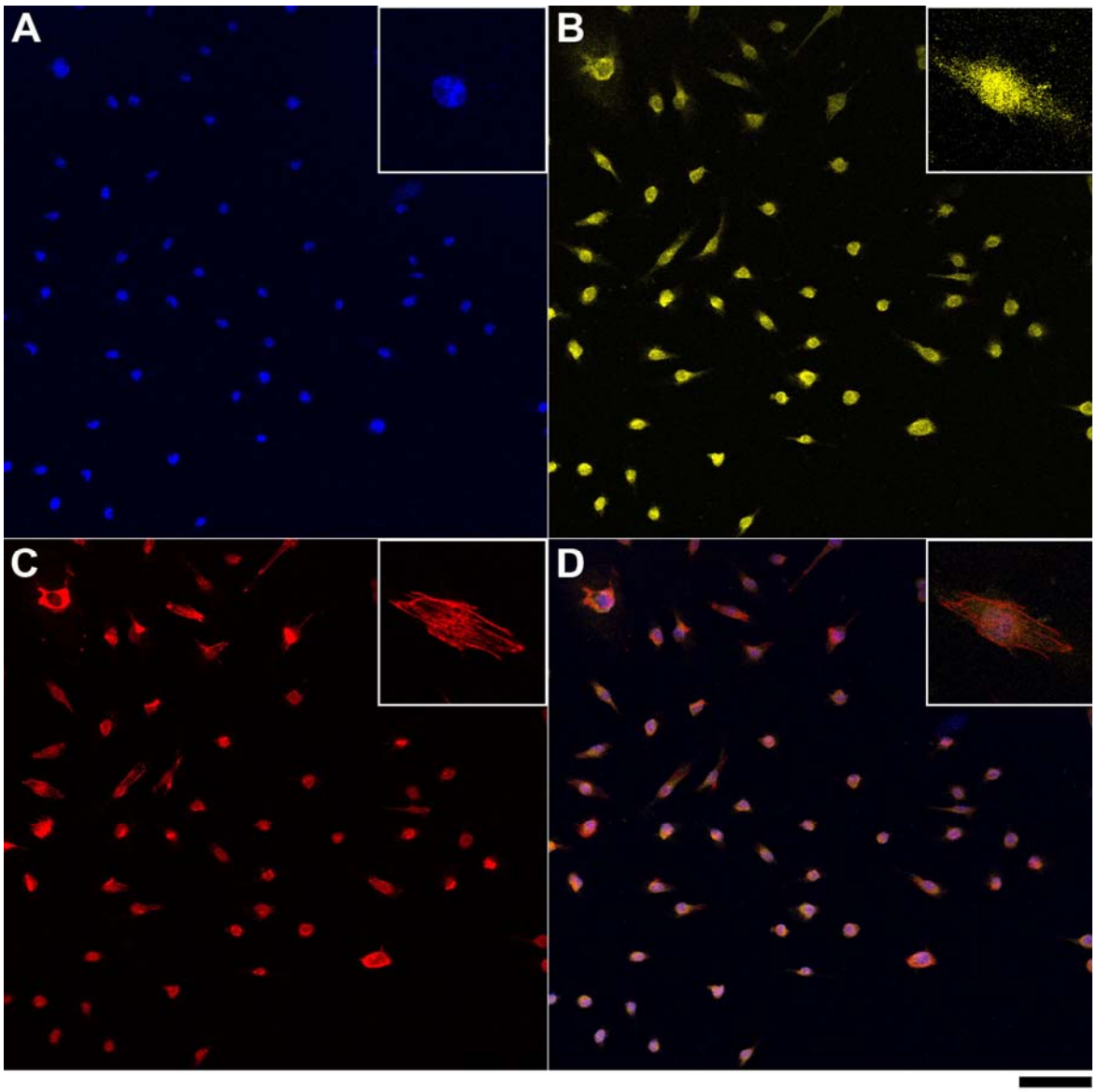


Figure S12. Fluorescent confocal microscope image shows that intracellular molecular aggregates curtail the efficacy of latruncunlin A (**4**) on F-actin depolymerization. The HeLa cells are pre-treated with 500 μM of **2a** in PBS buffer for one hour and then exposed to 10 μM of **4** for 30 minutes immediately before fixation by paraformaldehyde and fluorescence staining. (A) DAPI (blue) for nucleus; (B) NBD (yellow) from **3a**; (C) Alexa Fluor® 633 phalloidin (red) for F-actin and (D) merged image of A-C. Scale bar = 100 μm. Insets (75 μm × 75 μm) represent an individual cell at higher magnification.

S10.3. HeLa cells (pre-treated with compounds **2b-d**) visualized with F-actin / nuclei staining

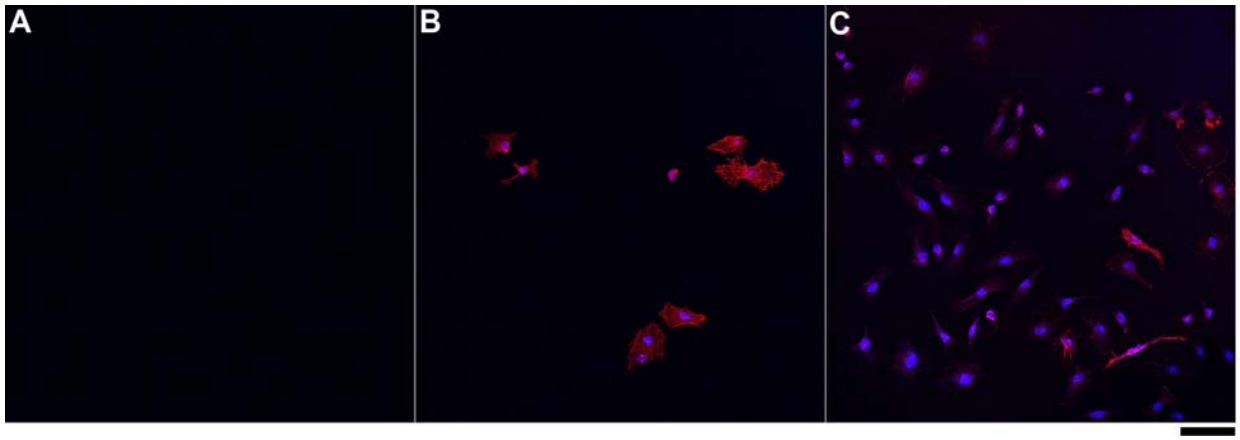


Figure S13. After a pre-treatment with 500 μM of (A) **2b**, (B) **2c**, and (C) **2d** in PBS buffer for one hour, each group of HeLa cells are exposed to 10 μM of **4** for 30 minutes immediately before fixation by paraformaldehyde. Each group of HeLa cells are then visualized under fluorescent microscope after the fluorescence staining with DAPI (nuclei) and Alex Fluor 633 Phalloidin (F-actin). Scale bar: 100 μm.

S11. The detailed procedure of the synthesis of **1**, **2a-d**

Synthesis of **1**: **1** was prepared by solid phase peptide synthesis (SPPS) using 2-chlorotrityl chloride resin (100~200 mesh, ~1.0 mmol/g) and N-Fmoc-protected amino acids with side chains properly protected or modified (Fmoc-Tyr(OPO_3H_2), Fmoc-Lys(Boc)-OH, Fmoc-Phe-OH) and 2-Naphthylacetic acid (Nap). 1 gram resin was firstly allowed swelling in dry dichloromethane (DCM) for 20 minutes with N_2 bubbling and then washed with dry N,N-dimethylformamide (DMF) (3 × 3 mL). Then the first amino acid was loaded onto resin at its C-terminal by bubbling the resin in a DMF solution of Fmoc-Tyr(OPO_3H_2) (using 2 equiv.) and N,N-diisopropylethylamine (DIPEA) for 1 hour. After washed with DMF (3 × 3 mL), the resin was blocked with a mixture of DCM/MeOH/DIPEA (80:15:5) for 30 min to deactivate the unreacted sites on resin. Then the resins were treated with 20% piperidine (in DMF) for 30 min to remove the Fmoc group, washed with DMF (5 × 3 mL) followed by coupling Fmoc-Lys(Boc)-OH (using 3 equiv.) to the amino group of Tyr on the resin using DIPEA/HBTU (3 equiv.) as the coupling reagent. The Fmoc deprotection (30 minutes) and amino acid coupling (1 hour) steps were repeated to elongate the peptide chain until Nap was loaded. At the final step, the peptide was cleaved with TFA (10 mL) for 2 hours and the resulted crude products were precipitated in dry diethyl ether, purified by RP C18 HPLC and applied to lyophilizer to afford the white solid product of **1**. ^{1}H NMR (400 MHz, DMSO-d_6): δ8.37 (1H, d), 8.27 (1H, d), 8.20 (2H, m), 8.12 (1H, d), 7.85–7.71 (3H, m), 7.59 (1H, s), 7.46 (2H, m), 7.30–7.00 (15H, m), 4.61–4.34 (3H, m), 4.14 (1H, m), 3.53 (2H, q), 3.15–3.01 (2H, m), 2.99–2.86 (2H, m), 2.84–2.63 (4H, m), 1.48–1.14 (4H, m), 1.08–0.79 (2H, m); ESI MS (*m*/*z*): $[M]^+$ calcd. for $C_{45}H_{50}N_5O_{10}P$, 851.33; found $[M+H]^+$, 852.49; found $[M-H]^-$, 850.54.

Synthesis of **2a**: To a solution of **1** (50.0 mg, 58.7 μmol) and Na_2CO_3 (12.4 mg, 117.4 μmol) in H_2O was added a solution of NBD-Cl (58.7 μmol) dissolved in MeOH. The mixture was stirred at 50 °C for 2 h, and then cooled to room temperature, neutralized with 10% HCl (aq). The solvent was removed under reduced pressure, and the crude product was purified by RP C18 HPLC (total yield: 67%). ^{1}H NMR (400 MHz, DMSO-d_6): δ9.54 (1H, s), 8.46 (1H, d), 8.38–8.01 (3H, m), 7.87–7.62 (3H, m), 7.61–7.50 (1H, d), 7.48–7.35 (2H, m), 7.28–6.92 (15H, m), 6.35 (2H, m), 4.69–4.12 (4H, m), 3.54 (2H, q), 3.07–2.63 (8H, m), 1.79–1.18 (6H, m); ESI MS (*m*/*z*): $[M]^+$ calcd. for $C_{51}H_{51}N_8O_{13}P$, 1014.33; found $[M+H]^+$, 1015.49; found

$[M-H]^-$, 1013.54.

Synthesis of **2b**: To a solution of **1** (50.0 mg, 58.7 μmol) and Na_2CO_3 (12.4 mg, 117.4 μmol) in H_2O was added a solution of DNS-Cl (58.7 μmol) dissolved in THF. The mixture was stirred at 60 °C for overnight, and then cooled to room temperature, neutralized with 10% HCl (aq). The solvent was removed under reduced pressure, and the crude product was purified by RP C18 HPLC (total yield: 87%). ^{1}H NMR (400 MHz, DMSO-d_6): δ8.45 (1H, d), 8.36 (1H, d), 8.29 (1H, d), 8.24 (1H, d), 8.16 (1H, d), 8.08 (1H, d), 7.89–7.75 (5H, m), 7.71 (1H, s), 7.65-7.50 (4H, m), 7.48-7.33 (13H, m), 7.25 (1H, d), 7.15 (1H, t), 7.02 (1H, t), 4.34–4.16 (3H, m), 4.07 (1H, m), 3.53 (2H, q), 3.17–3.03 (2H, m), 2.99–2.87 (2H, m), 2.84–2.58 (4H, m), 2.82 (6H, s), 1.48–0.96 (6H, m); ESI MS (*m*/*z*): $[M]^+$ calcd. for $C_{57}H_{61}N_6O_{12}PS$, 1084.38; found $[M+H]^+$, 1085.49; found $[M-H]^-$, 1083.52.

Synthesis of **2c**: To a solution of **1** (50.0 mg, 58.7 μmol) and Na_2CO_3 (12.4 mg, 117.4 μmol) in H_2O was added a solution of DBD-F (58.7 μmol) dissolved in acetonitrile. The mixture was stirred at 60 °C for 3 h, and then cooled to room temperature, neutralized with 10% HCl (aq). The solvent was removed under reduced pressure, and the crude product was purified by RP C18 HPLC (total yield: 81%). ^{1}H NMR (400 MHz, DMSO-d_6): δ8.46 (1H, m), 8.30 (1H, m), 8.18 (1H, d), 8.08 (1H, m), 7.86–7.69 (3H, m), 7.58 (1H, d), 7.49-7.40 (2H, m), 7.36-6.88 (17H, m), 6.26 (1H, d), 4.64–4.22 (4H, m), 3.52 (2H, q), 3.08–2.58 (8H, m), 2.66 (6H, s), 1.73–1.11 (6H, m); ESI MS (*m*/*z*): $[M]^+$ calcd. for $C_{53}H_{57}N_8O_{13}PS$, 1076.35; found $[M+H]^+$, 1077.54; found $[M-H]^-$, 1075.43.

Synthesis of **2d**: To a solution of **1** (50.0 mg, 58.7 μmol) and Na_2CO_3 (12.4 mg, 117.4 μmol) in H_2O was added a solution of (5/6) TAMRA-SE (58.7 μmol) dissolved in DMF. The mixture was stirred at RT for overnight. The solvent was removed under reduced pressure, and the crude product was purified by RP C18 HPLC (total yield: 60%). ^{1}H NMR (400 MHz, DMSO-d_6): δ8.89 (1H, d), 8.77 (1H, d), 8.65 (1H, d), 8.55-8.09 (3H, m), 7.83 (3H, m), 7.74 (3H, m), 7.56 (1H, s), 7.46 (3H, m), 7.33–7.03 (15H, m), 7.03-6.81 (4H, m), 4.72–4.38 (3H, m), 4.28 (1H, m), 3.58 (2H, q), 3.20 (12H, s), 3.09–2.85 (4H, m), 2.84–2.56 (4H, m), 1.41–0.93 (6H, m); ESI MS (*m*/*z*): $[M]^+$ calcd. for $C_{70}H_{70}N_7O_{14}P$, 1263.47; found $[M+H]^+$, 1264.72; found $[M-H]^-$, 1262.67.