Supporting information for

Enzymatic Self-Assembly of Short Peptides for Cell Spheroid Formation

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Methods

Materials

2-Cl-trityl chloride resin and Fmoc protected amino acids were purchased from GL Biochem; Fmoc-Ser(PO(OBzl)OH)-OH from Sigma-Aldrich; biphenyl-4-carboxylic acid from Alfa Aesar; 4’-hydroxy-[1,1’-biphenyl]-4-carboxylic acid from 1PlusChem; 4'-Trifluoromethyl-biphenyl-4-carboxylic acid, 4'-Methylbiphenyl-4-carboxylic acid, 4'-Chloro-biphenyl-4-carboxylic acid, and 4'-Nitrobiphenyl-4-carboxylic acid from AK Scientific; HBTU from Chem Impex; DIPEA from TCI America; alkaline phosphatase from Biomatik; recombinant human PAP protein (Active) from Abcam (ab219224). Rhodamine-fibronectin was purchased from Cytoskeleton. Tubulin Tracker™ Deep Red was purchased from Invitrogen. Hoechst 33342 was purchased from Molecular Probes. Organic solvents and other chemicals were purchased from Thermo Fisher. All materials were used as received without further purification.

Cell culture supplies such as 35 mm Glass bottom dish with 20 mm micro-well #1.5 cover glass was purchased from Cellvis; 100 mm cell culture dish from Cell Treat; Fetal bovine serum (FBS), Minimum Essential Medium (MEM), and penicillin-streptomycin (PS) from Gibco; Dulbecco's Modified Eagle Medium (DMEM), RPMI 1640 medium from American Type Culture Collection (ATCC, USA).

Instruments

Purification was performed using reverse phase HPLC (Agilent 1100 Series), eluted with HPLC grade acetonitrile and 10 mM ammonium acetate buffered HPLC grade water. LC-MS spectra were obtained on a Waters Acquity Ultra Performance Liquid Chromatography equipped with Waters Micromass Quattro Mass Spectrometer. TEM was performed on Morgagni 268. Circular dichroism was performed on Jasco J-810 spectropolarimeter. Absorbance spectra were obtained on BioTek UV/Vis/fluorescence plate reader. Phase contrast images were taken on a Nikon TS100 Inverted Phase

Contrast Microscope. The absorbance at 595 nm for MTT assays was measured by Beckman DTX880 Multimode Detector.

Peptide synthesis (BP-L4$_p$Y as an example)

Peptides were synthesized according to standard solid-phase synthesis using 2-chlorotrityl resin (Scheme S1). Fmoc-O-phospho-L-tyrosine was synthesized according to reference.[1] The peptides were cleaved using a cleavage cocktail (95% TFA, 2.5% triisopropyl silane, and 2.5% H_2O) at room temperature for 1 h. Peptides were concentrated, precipitated with ice-cold ethyl ether, and then purified by HPLC.

Determination of critical micelle concentration (CMC)

The CMCs were measured using rhodamine 6G, an environment-sensitive probe. Various sample concentrations were prepared in PBS with 5 μM rhodamine 6G. The maximum absorption wavelength was plotted against the peptide concentration, and the CMC was determined at the intersection point of two linear regression lines.

Transmission electron microscopy

Copper grids with a 400-mesh and carbon film were glow discharged at -20 mA for 30 seconds. A 5 μL sample was then placed on each grid and allowed to sit for 1 minute before removing the excess with filter paper. The grids were stained twice with 2% uranyl acetate for 20 seconds per stain, and any excess stain was blotted away with filter paper. Once fully dried, the grids were imaged at 80 kV high tension.

Cell culture

HS-5, HeLa, and SJSA-1 cells were purchased from ATCC, OVSAHO was a gift from D. Dinulescu. HS-5, and HeLa cell lines were authenticated by CellCheck 9 - human (9 Marker STR Profile and Inter-species Contamination Test, IDEXX), confirming 100%

match of the cell identity. HS-5 cells were cultured in DMEM supplemented with 10% FBS. HeLa cells were cultured in MEM supplemented with 10% FBS. SJSA-1 and OVSAHO cells were cultured in RPMI 1640 medium supplemented with 10% FBS. All the cell lines were supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin and were cultured and humidified with 5% CO2 at 37 ℃.

Spheroid formation in HS-5 cells

Adherent cells

Cells were plated at a density of 1.5×10^5 cells per dish on the inner 200 mm glass surface of a 3.5 cm confocal dish and allowed to adhere for 24 hours. A fresh culture medium (500 μL) containing the peptide at the working concentration was prepared directly from a 10 mM stock solution in PBS. Phase contrast images were taken after incubating the cells with the peptide-containing medium for a defined period.

Suspended cells

Cells were trypsinized to form a suspension at a concentration of 1.5×10^5 cells/mL in culture medium. A fresh 10 mM peptide stock solution was directly added to the medium to create a working cell suspension. Around 7.5×10^4 cells were then seeded onto the 200 mm inner glass surface of 3.5 cm confocal dishes for incubation. Phase contrast images were obtained after the cells were incubated with the culture medium for a specified period.

Circular Dichroism (CD)

CD spectra were obtained in the 190−250 nm range using a JASCO 8-10 spectrometer in a nitrogen atmosphere. Peptide was introduced into a 1 mm quartz cuvette and scanned at 1 nm intervals three times, with the average values of the three measurements being recorded.

The free energy of micellization was calculated according to the following equation:

$$\Delta G_{(micellization)} = RT\ln(CMC)$$

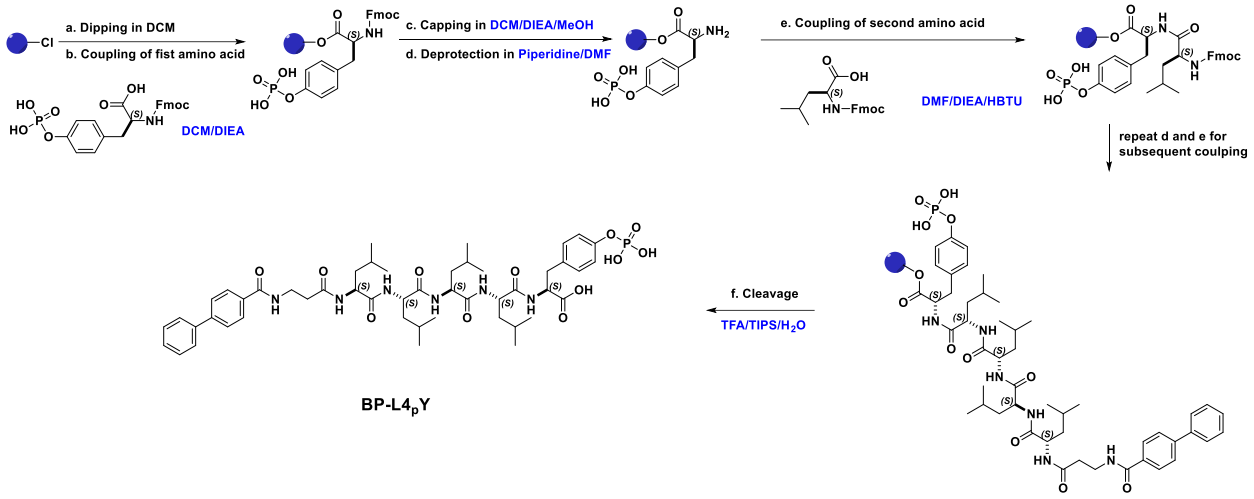
Determination of dephosphorylation by ALP

Peptides (400 μM) were incubated with ALP (0.1 U/mL) in PBS for 48 h and then quenched with equal volume of methanol. The mixture was injected into an HPLC to determine the enzymatic dephosphorylation of peptides.

MTT assay

Cells were seeded at a density of 10^4 cells per well in 96-well plates and given 24 hours to adhere. The culture media were then replaced with fresh media containing varying concentrations of the compounds. After incubation periods of 24, 48, or 72 hours, 10 μL of MTT solution (5 mg/mL) was added to each well, and the plates were incubated in the dark at 37°C for 4 hours. To stop the reaction and dissolve the formazan crystals, 100 μL of 10% SDS-HCl was added to each well. Absorbance at 595 nm was measured using a microplate reader. The assay was performed in triplicate to calculate the average values from three independent measurements.

Supporting Schemes



Scheme S1. Synthetic route of BP-L4$_p$Y.

Supporting Figures

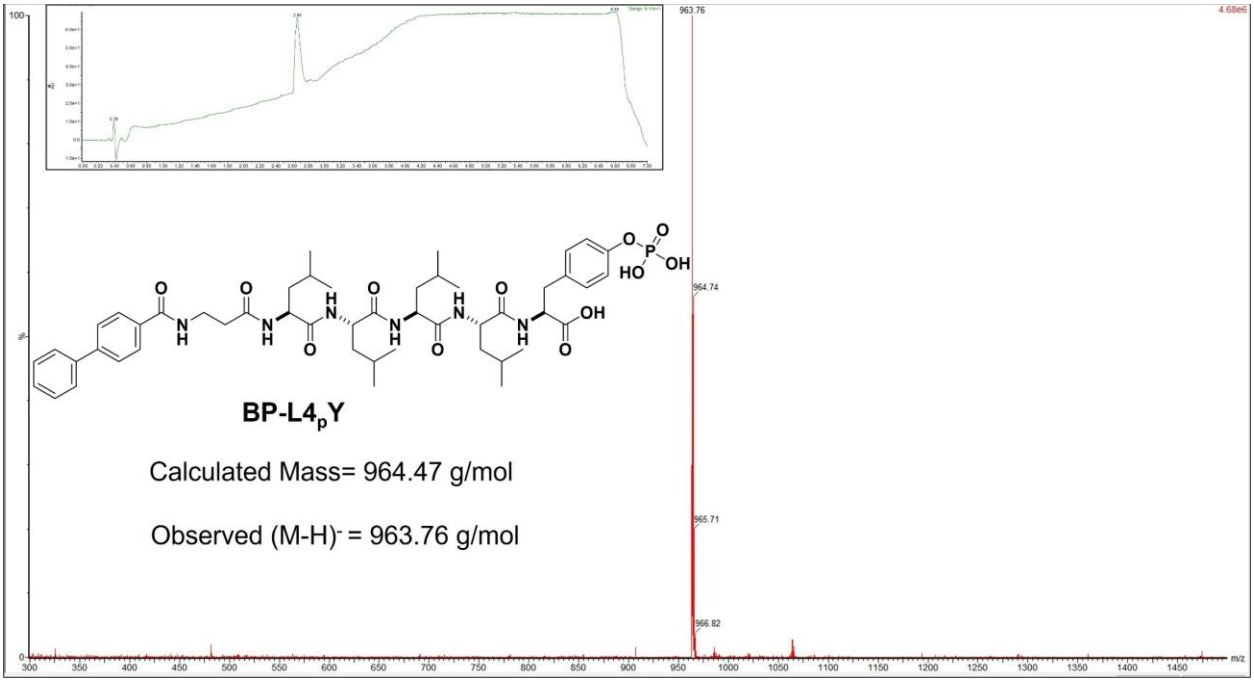


Figure S1. LC-MS spectra of BP-L4$_p$Y. Inset is the LC spectrum.

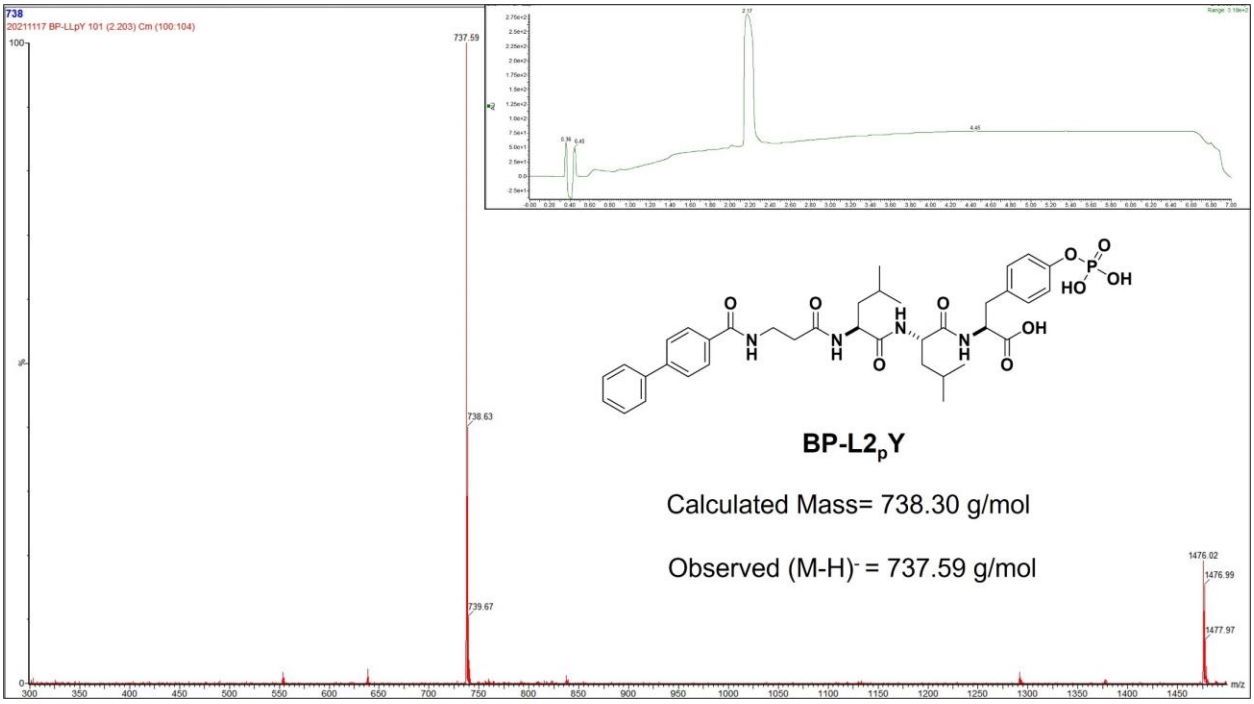


Figure S2. LC-MS spectra of BP-L2$_p$Y. Inset is the LC spectrum.

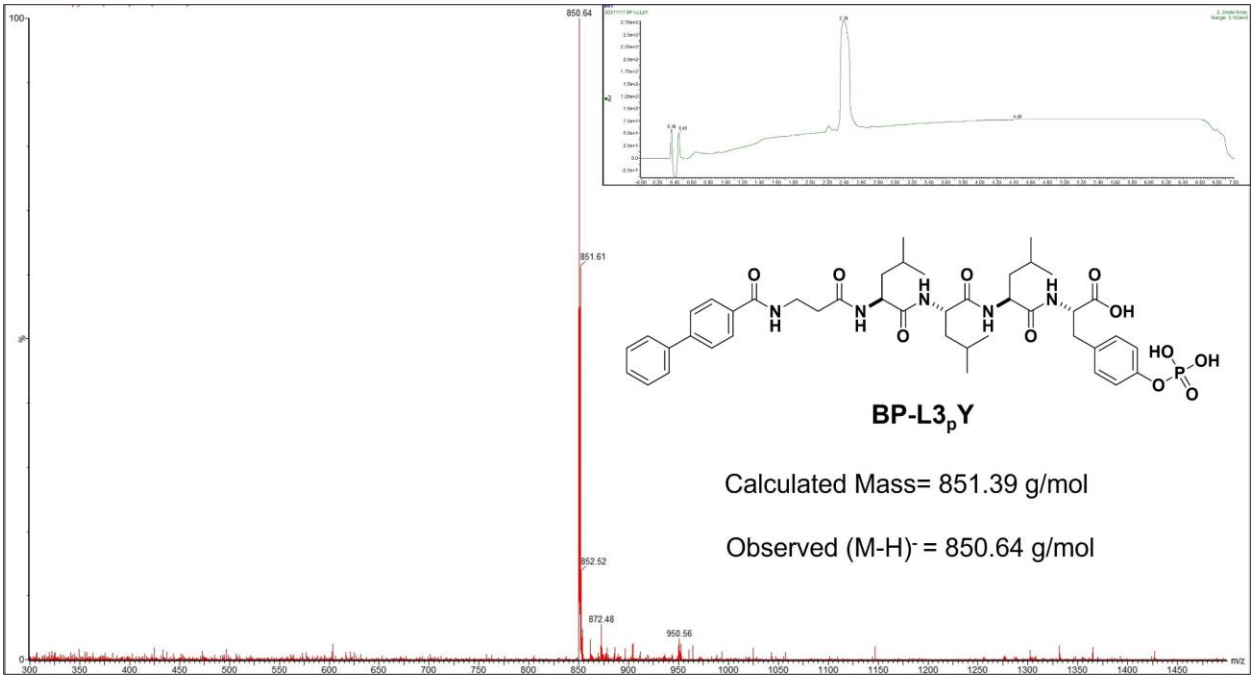


Figure S3. LC-MS spectra of BP-L3$_p$Y. Inset is the LC spectrum.

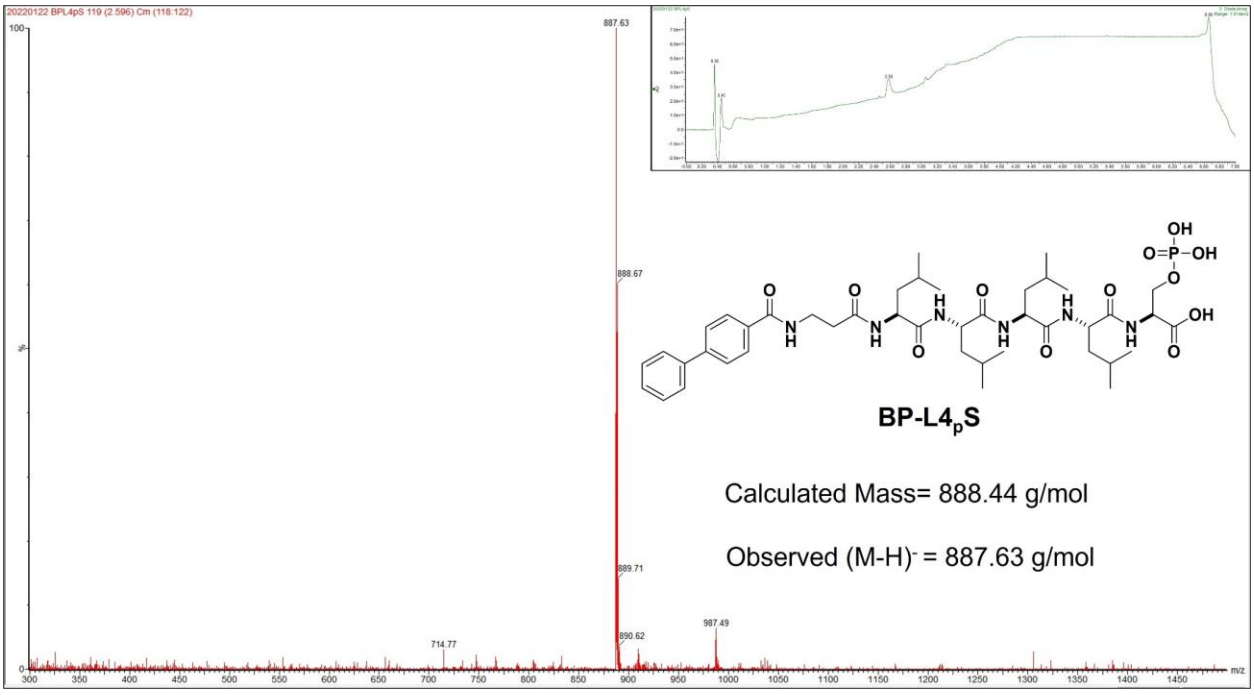


Figure S4. LC-MS spectra of BP-L4$_p$S. Inset is the LC spectrum.

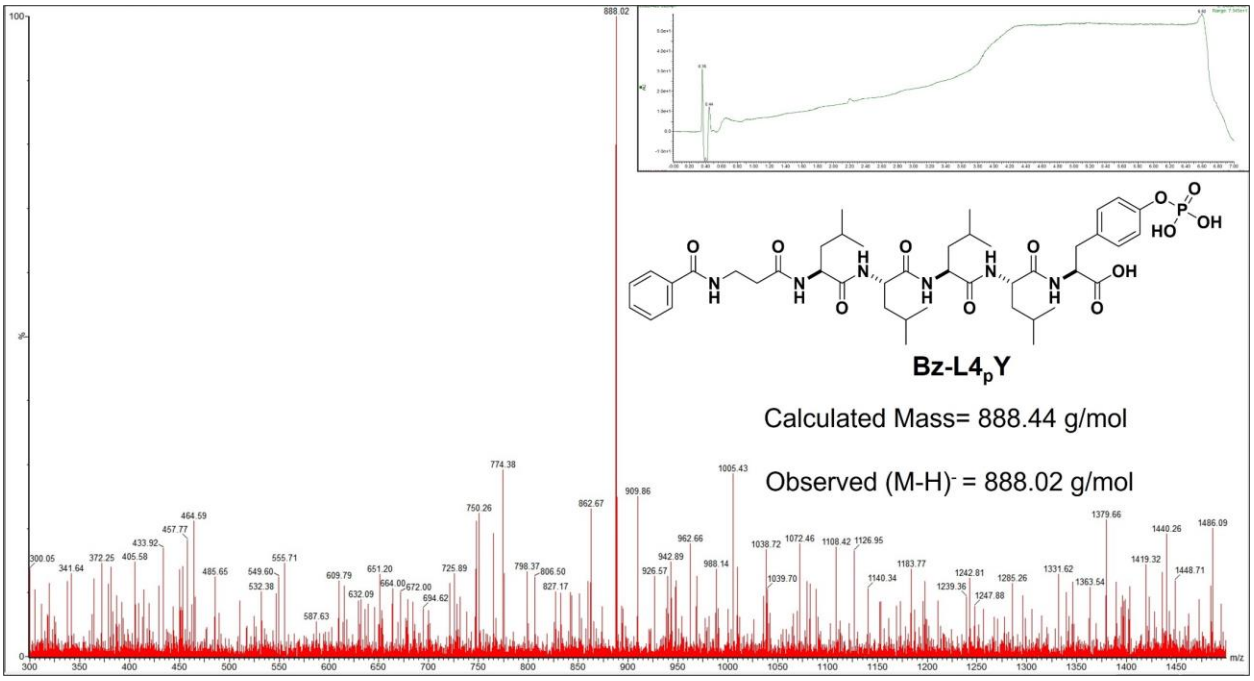


Figure S5. LC-MS spectra of Bz-L4$_p$Y. Inset is the LC spectrum.

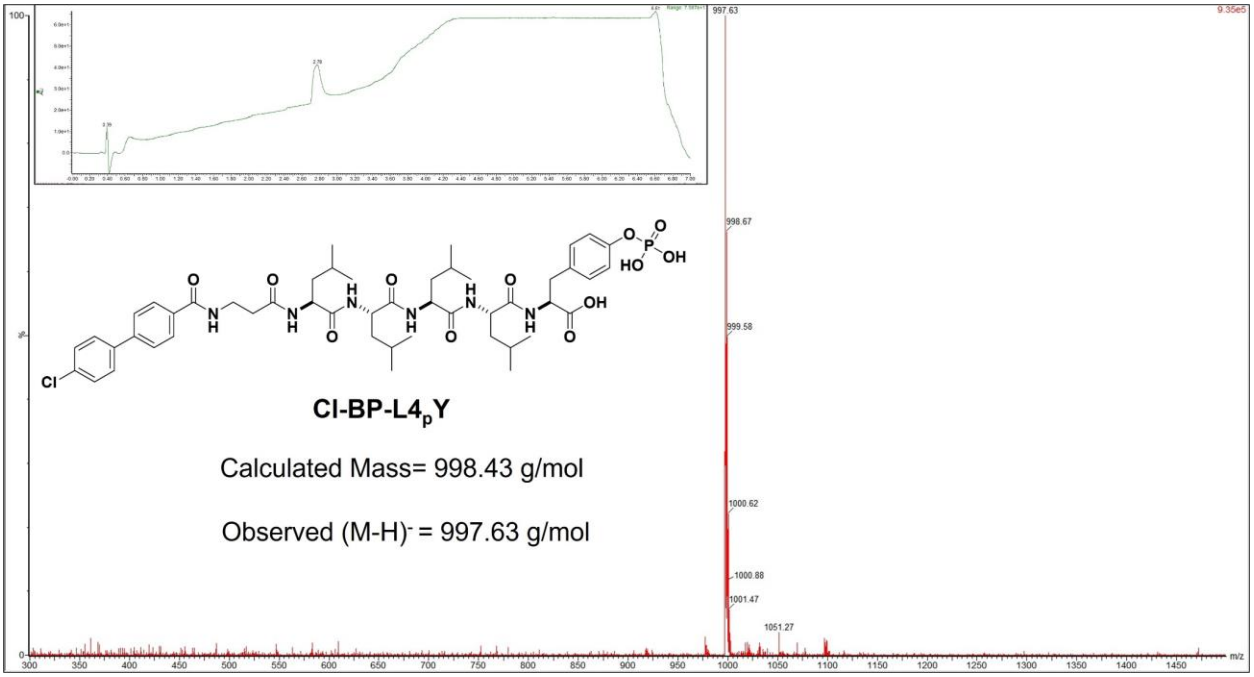


Figure S6. LC-MS spectra of Cl-BP-L4$_p$Y. Inset is the LC spectrum.

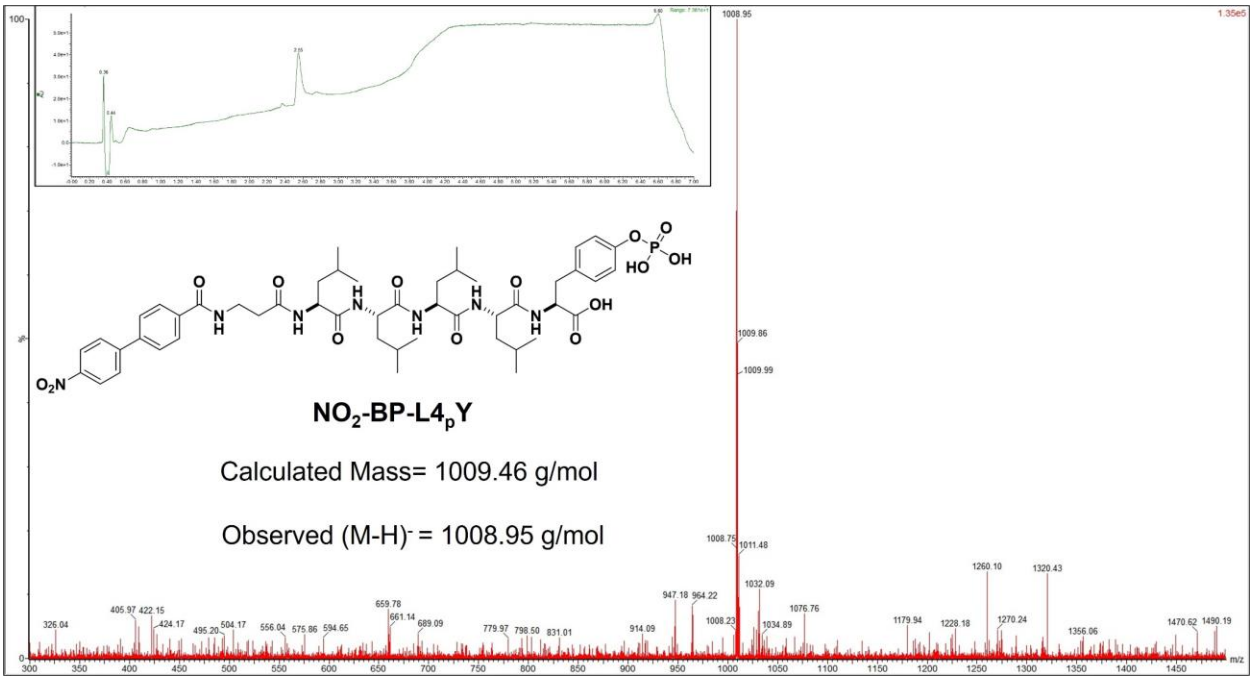


Figure S7. LC-MS spectra of NO_2-BP-L4$_p$Y. Inset is the LC spectrum.

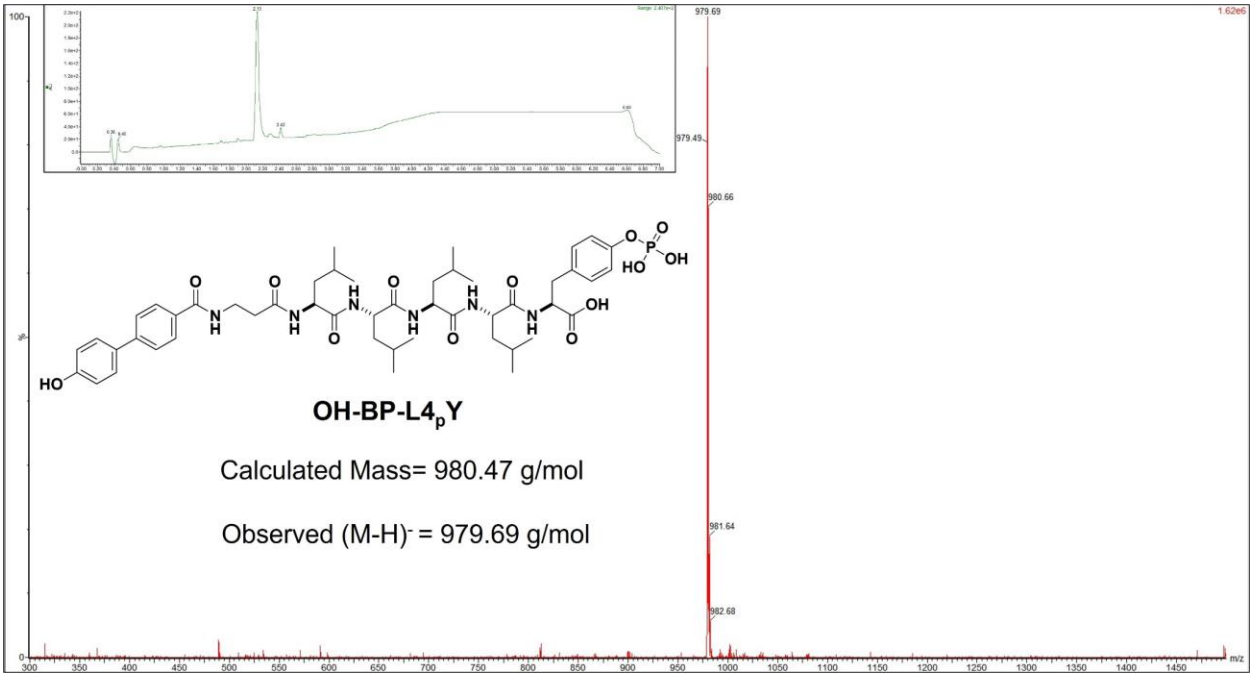


Figure S8. LC-MS spectra of OH-BP-L4$_p$Y. Inset is the LC spectrum.

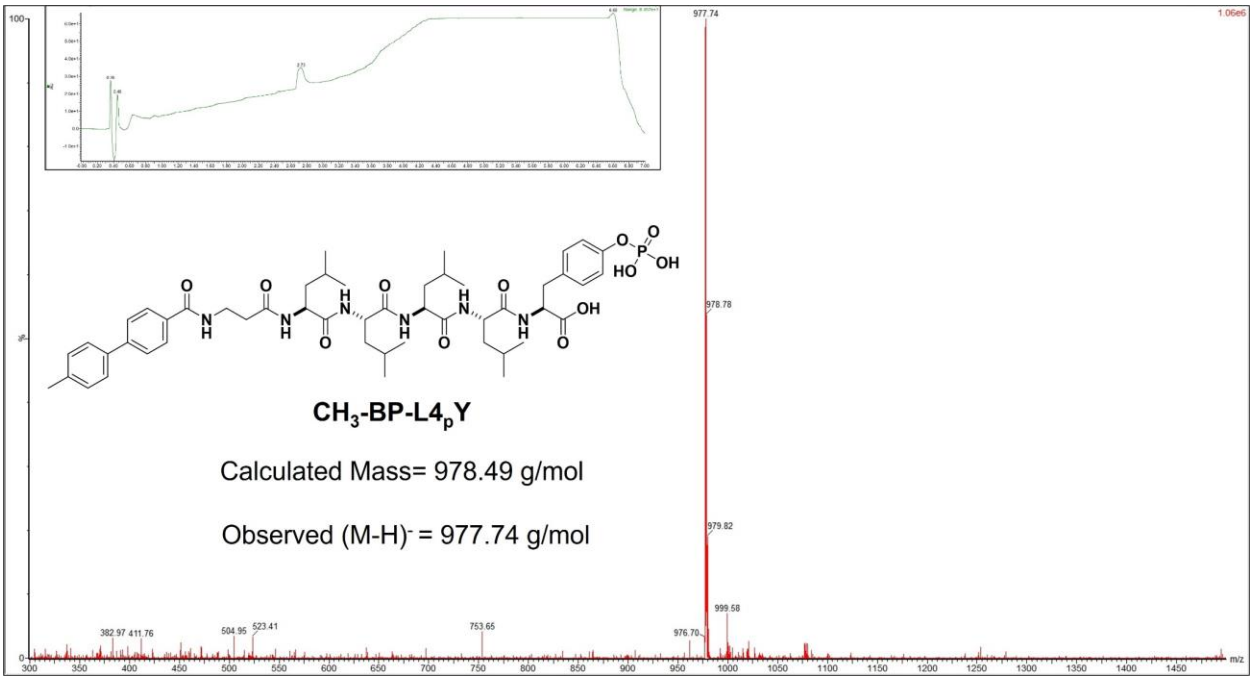


Figure S9. LC-MS spectra of CH_3-BP-L4$_p$Y. Inset is the LC spectrum.

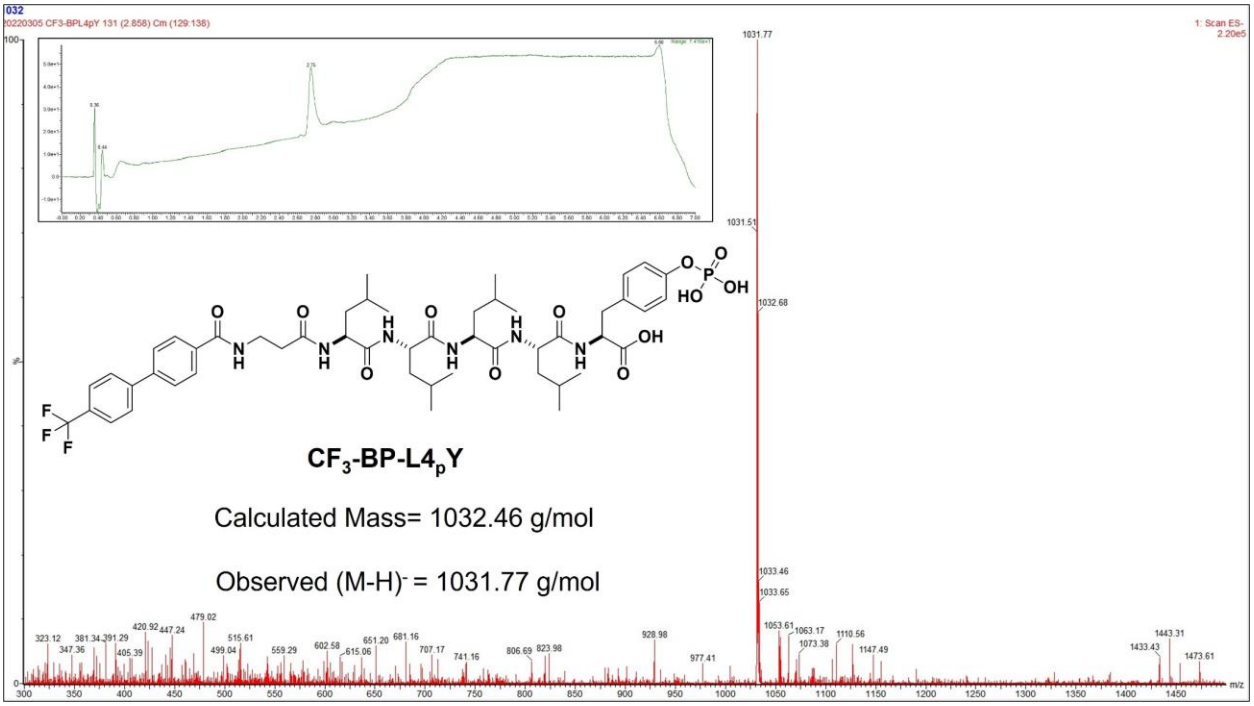


Figure S10. LC-MS spectra of CF_3-BP-L4$_p$Y. Inset is the LC spectrum.

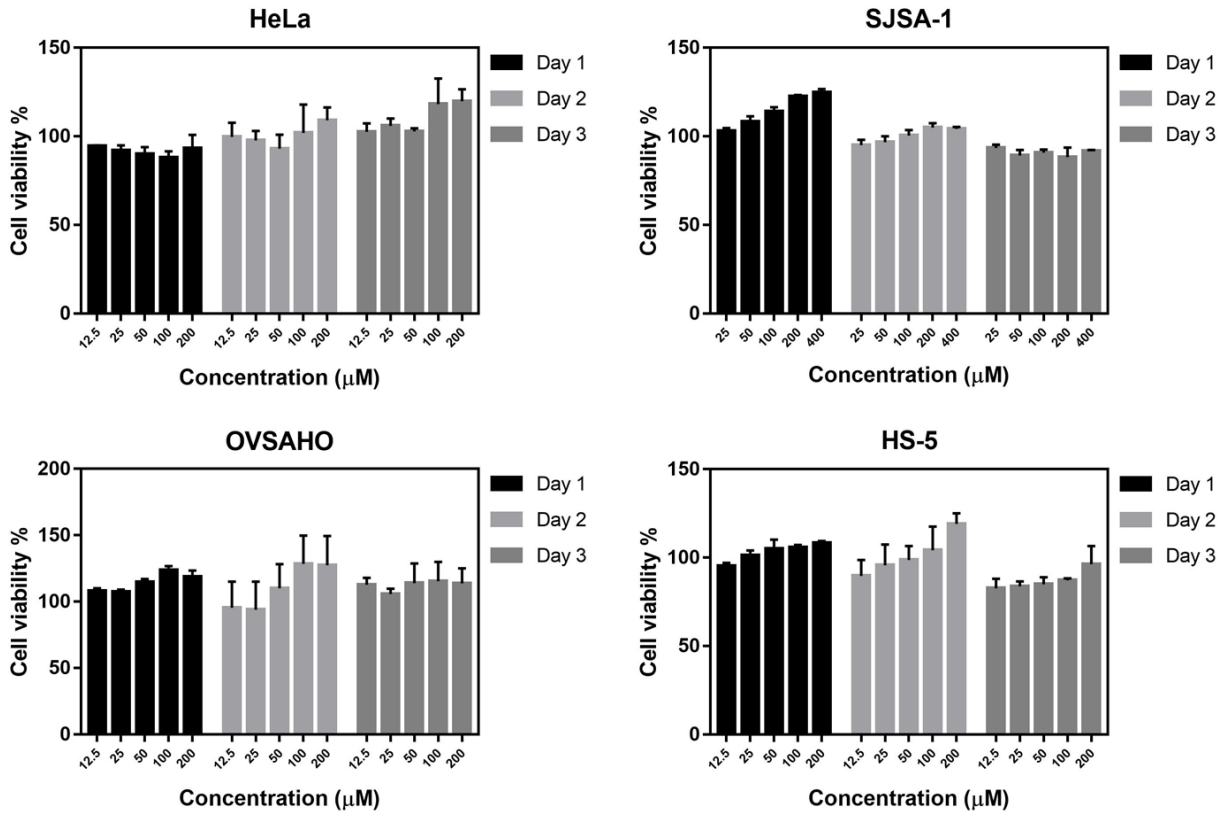


Figure S11. Cytotoxicity of BP-L4$_p$Y on different cell lines. Incubation time is 24, 48, or 72 h.

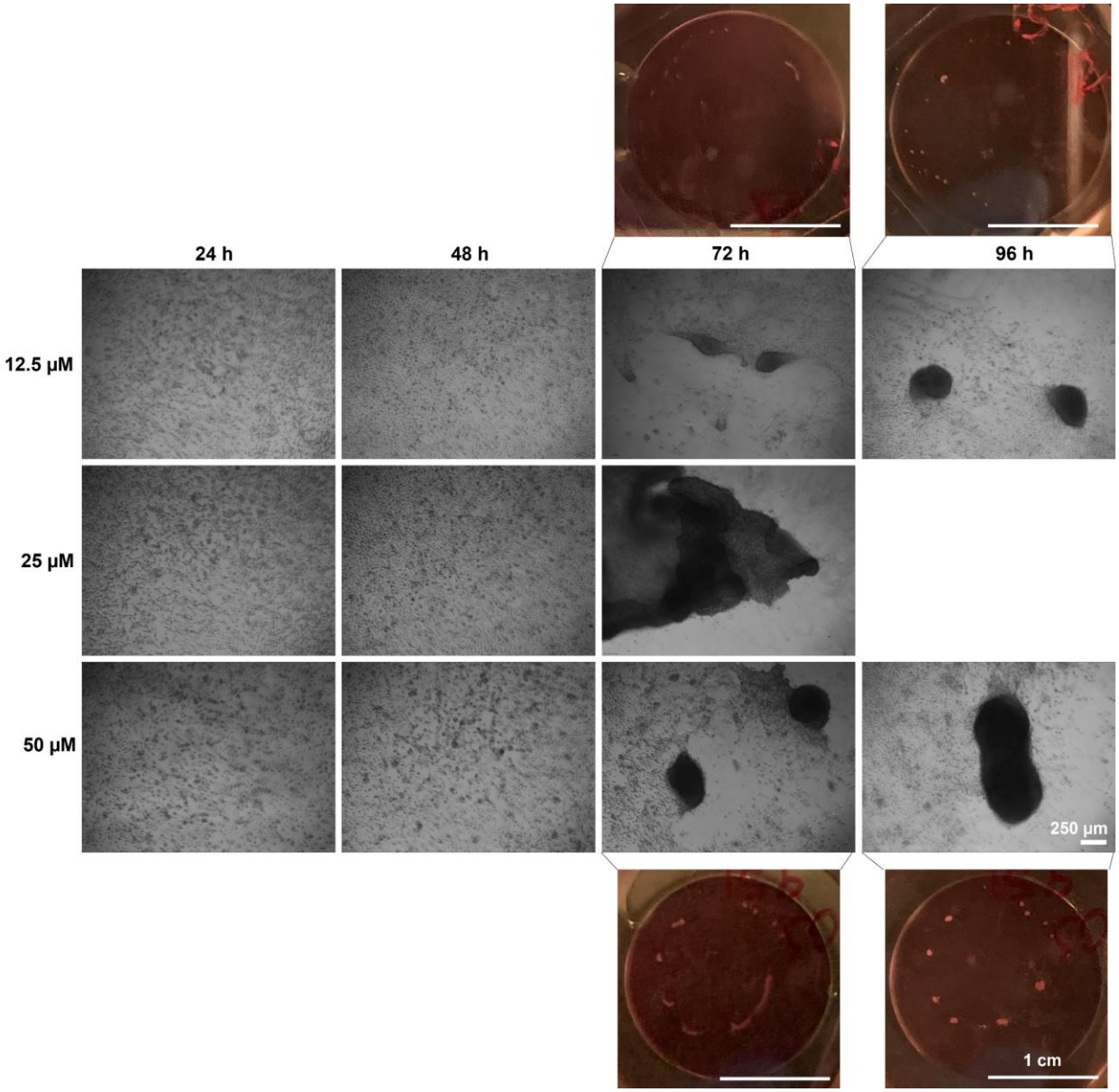


Figure S12. Phase contrast and optical images of adherent HS-5 cells treated with BP-L4$_p$Y (12.5 μM-50 μM) at designated time points.

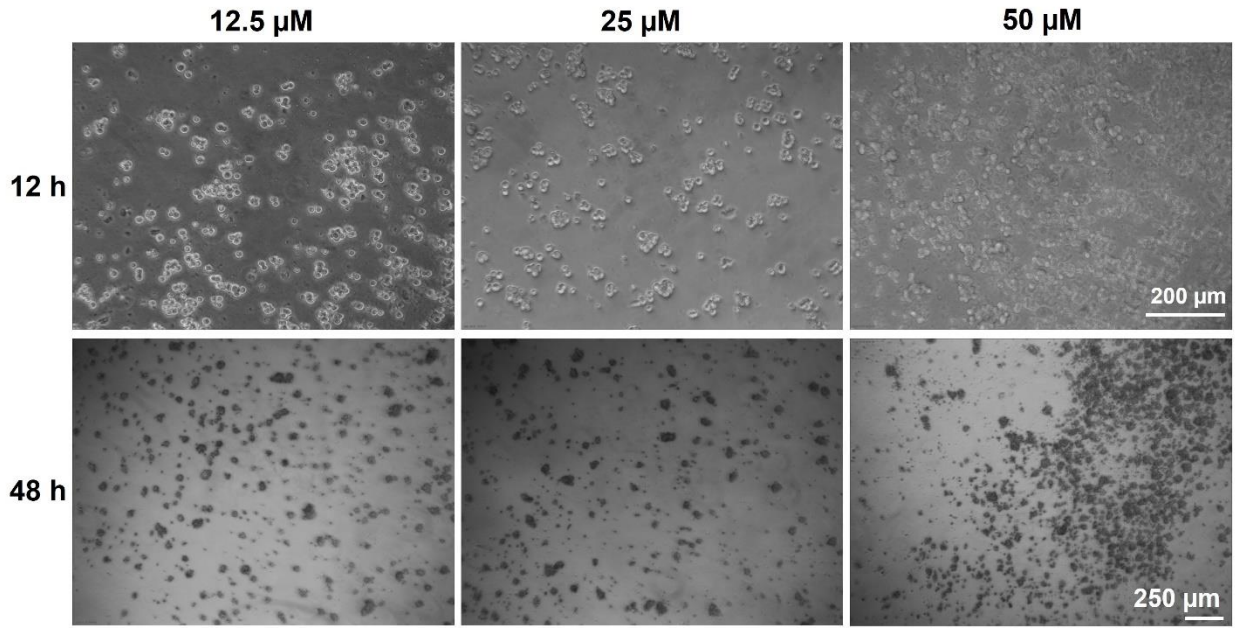


Figure S13. Phase contrast images of suspended OVSAHO cells treated with BP-L4$_p$Y (12.5-50 μM) for 12 or 48 h.

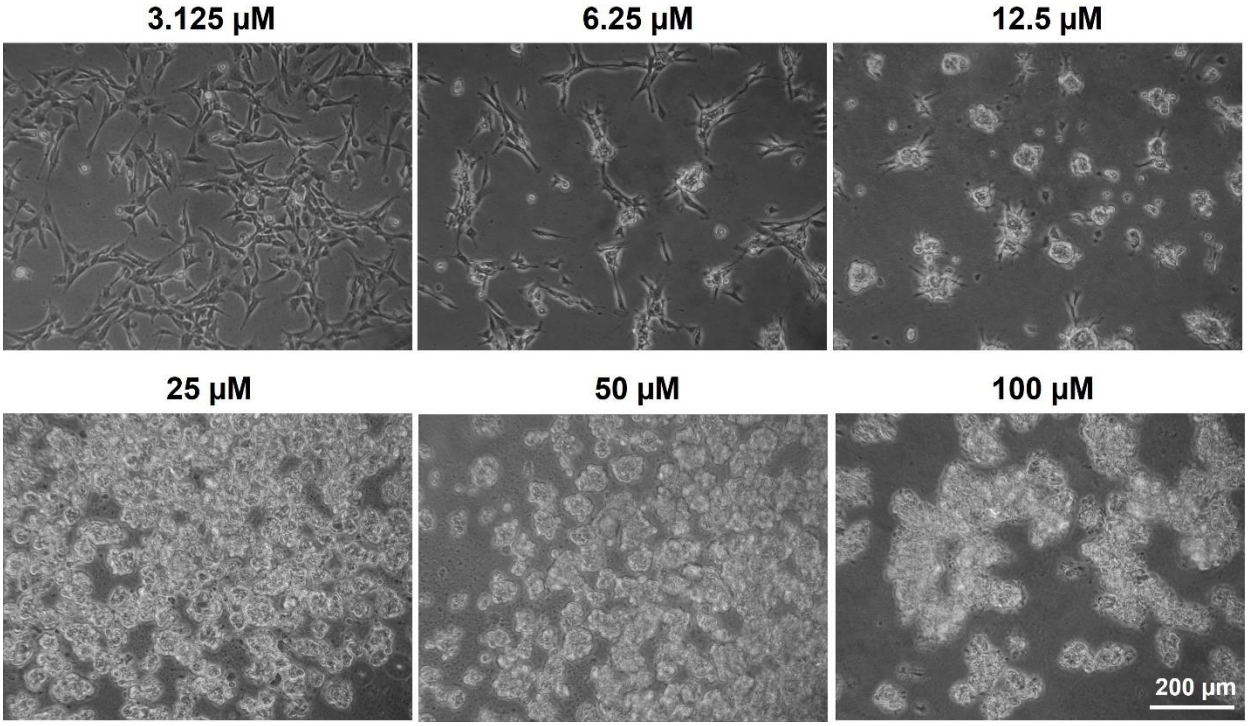


Figure S14. Phase contrast images of suspended SJSA-1 cells treated with BP-L4$_p$Y (3.125-100 μM) for 12 h.

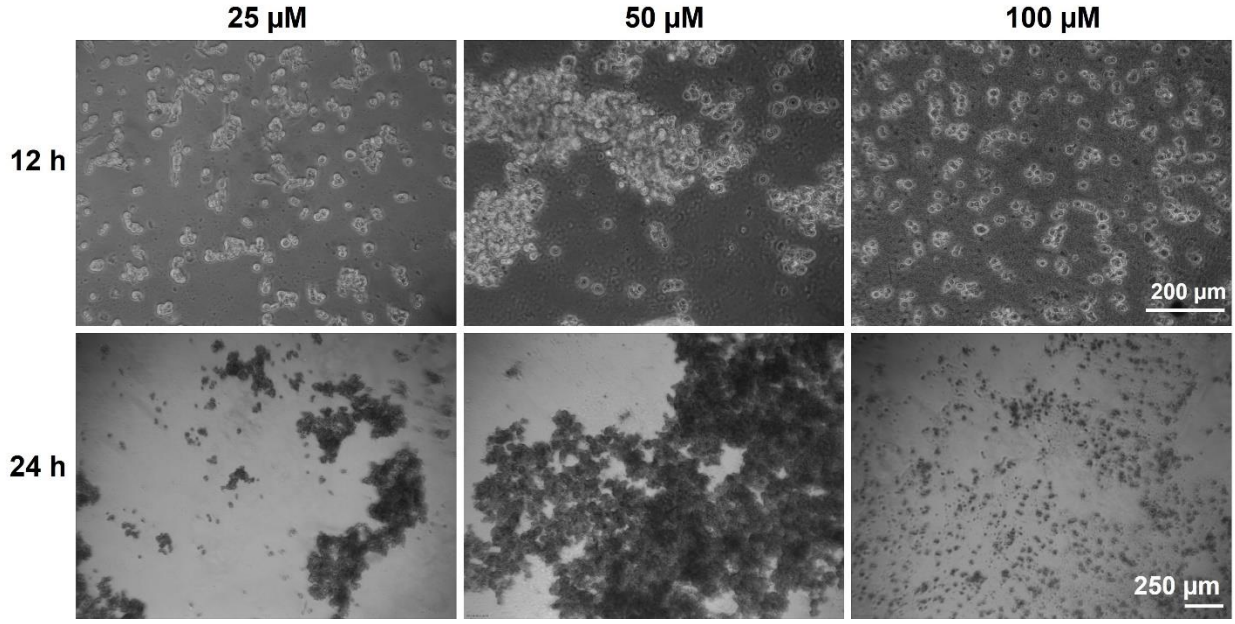


Figure S15. Phase contrast images of suspended HeLa cells treated with BP-L4$_p$Y (25-100 μM) for 12 or 24 h.

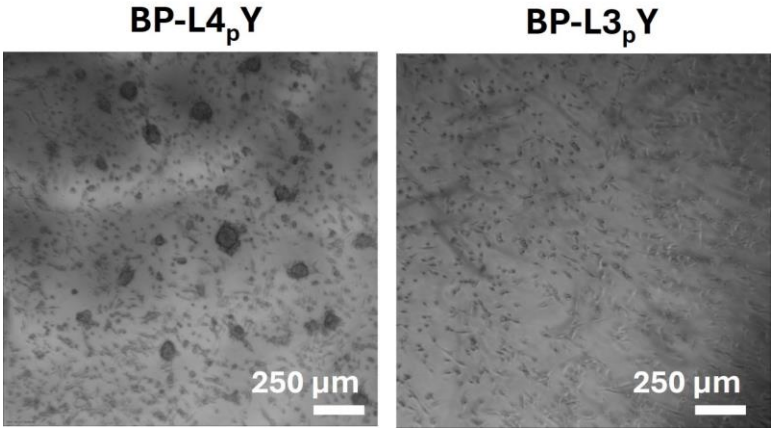


Figure S16. Phase contrast images of suspended HS-5 cells treated with BP-L4$_p$Y or BP-L3$_p$Y (3.13 μM) for 48 h.

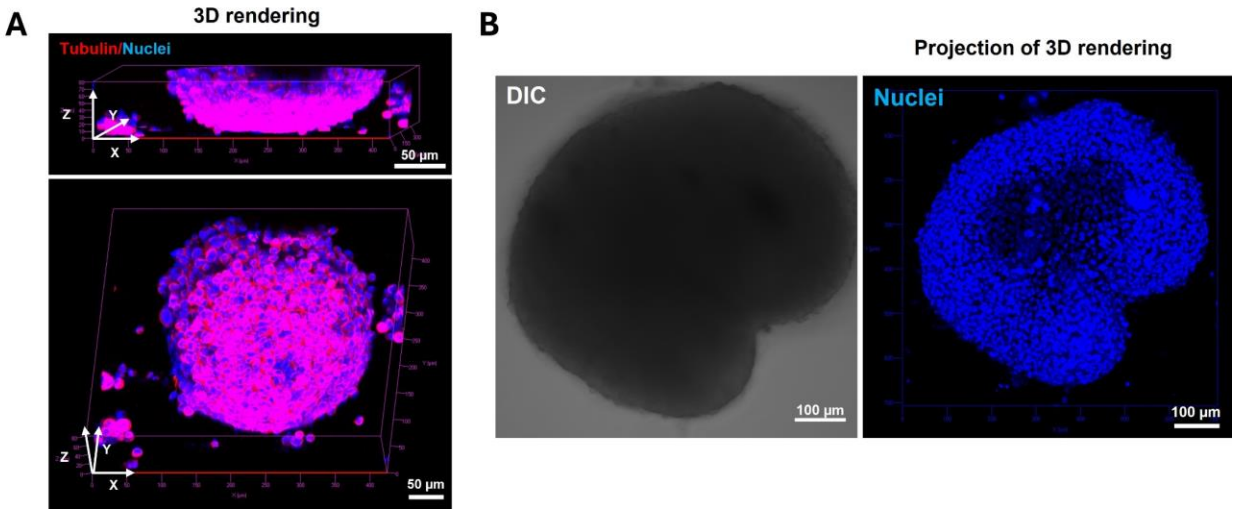


Figure S17. (A) 3D rendering confocal images of HS-5 spheroid stained with Tubulin Tracker and Hoechst for 1 h. (B) DIC and the projection of Z-stack images of HS-5 spheroid formed by incubating with BP-L4$_p$Y (13 μM) for 72 h and then stained with Hoechst for 1 h.

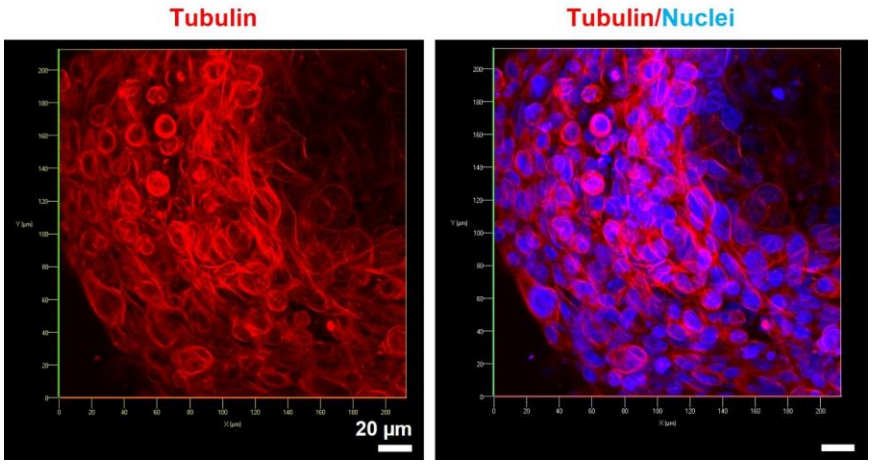


Figure S18. Projection Z-stack images of spheroid stained with Tubulin Tracker and Hoechst for 1 h.

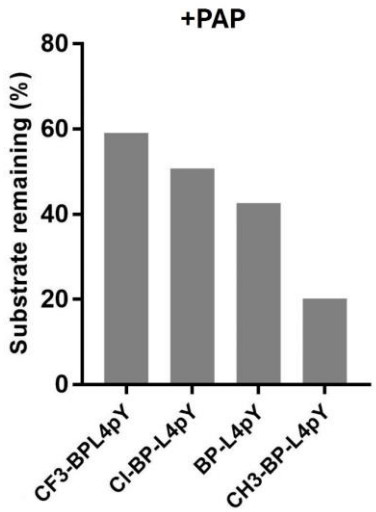


Figure S19. Enzymatic conversion of BP-L4$_p$Y and analogs (400 μM) in the treatment of PAP (0.1 U/mL) for 48 h in PBS.

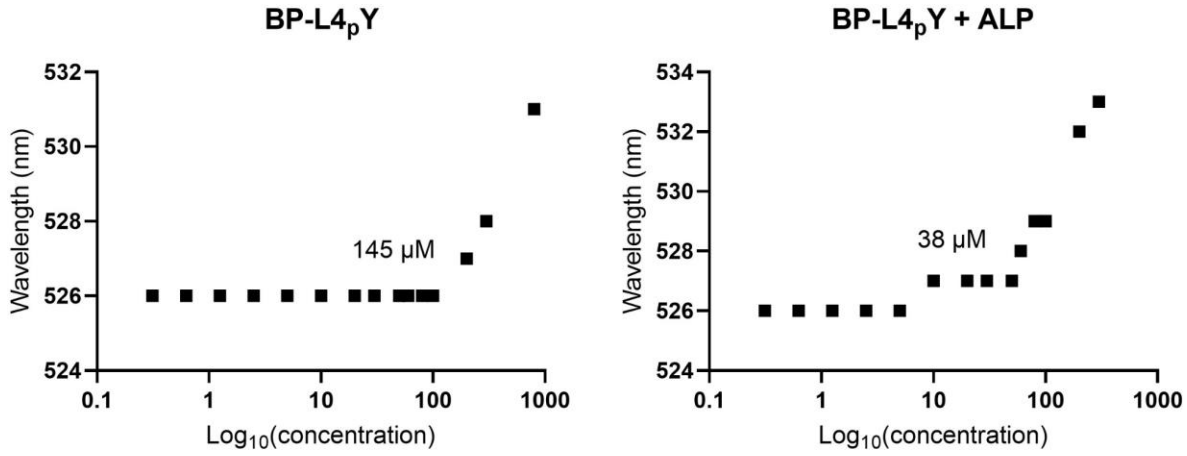


Figure S20. CMC determination of BP-L4$_p$Y in the absence or presence of ALP (0.1 U/mL) for 48 h in PBS.

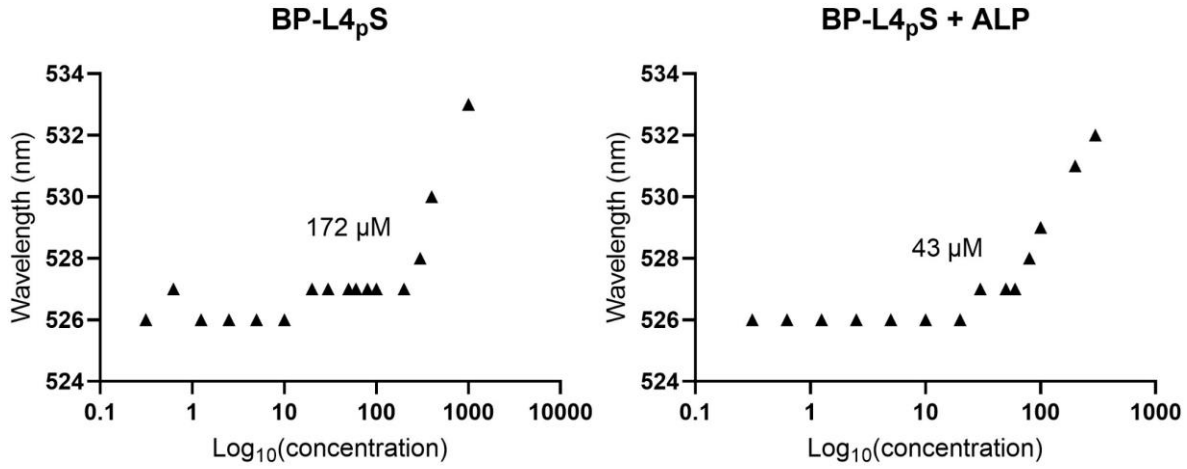


Figure S21. CMC determination of BP-L4$_p$S in the absence or presence of ALP (0.1 U/mL) for 48 h in PBS.

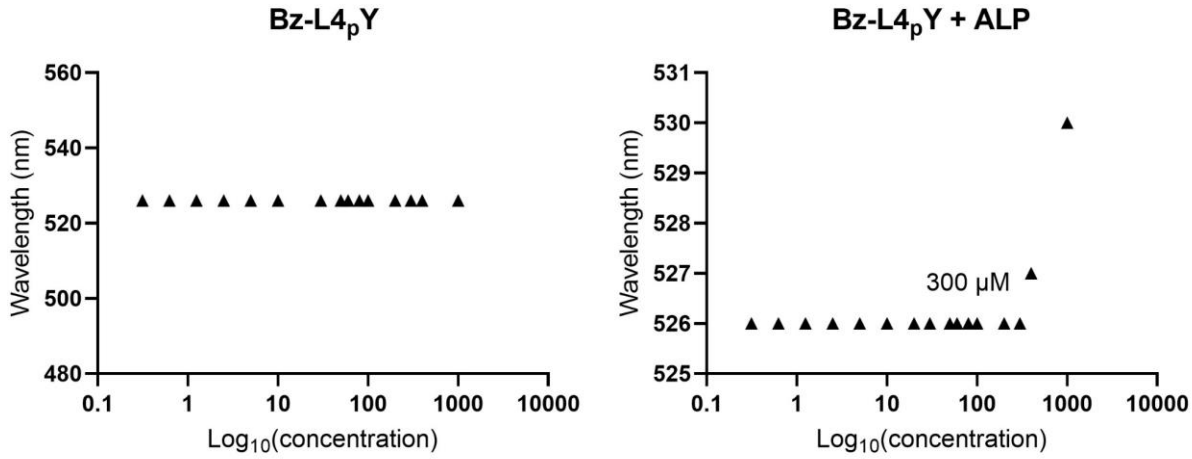


Figure S22. CMC determination of Bz-L4$_p$Y in the absence or presence of ALP (0.1 U/mL) for 48 h in PBS.

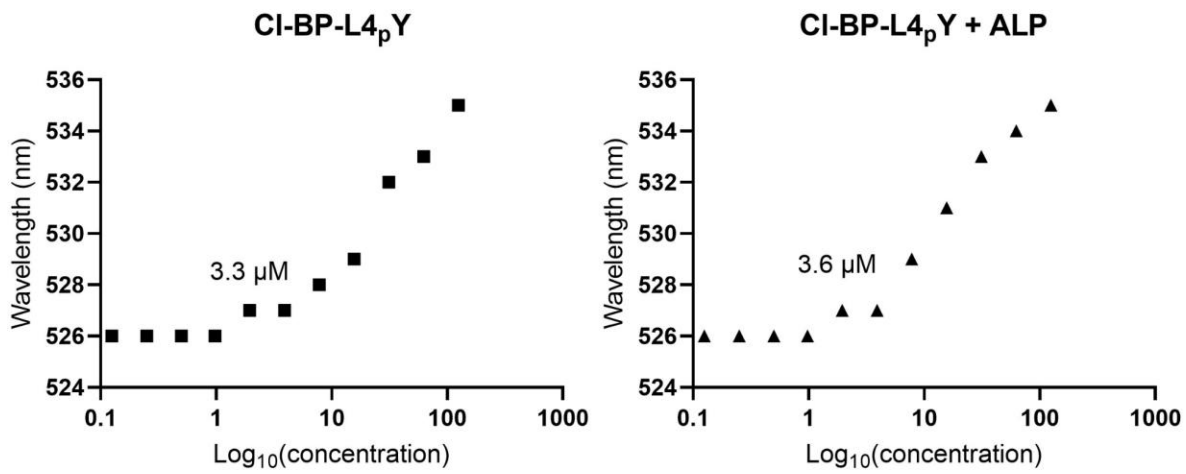


Figure S23. CMC determination of Cl-BP-L4$_p$Y in the absence or presence of ALP (0.1 U/mL) for 48 h in PBS.

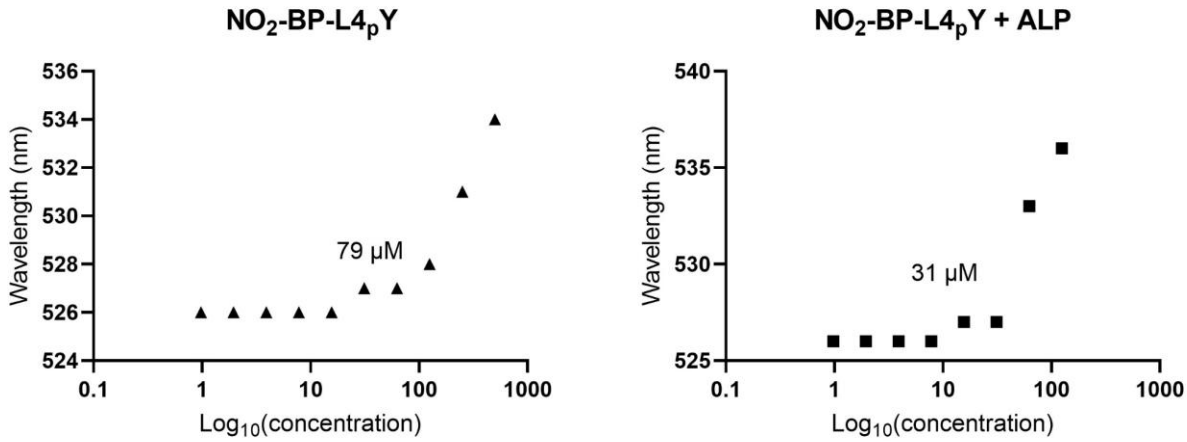


Figure S24. CMC determination of NO_2-BP-L4$_p$Y in the absence or presence of ALP (0.1 U/mL) for 48 h in PBS.

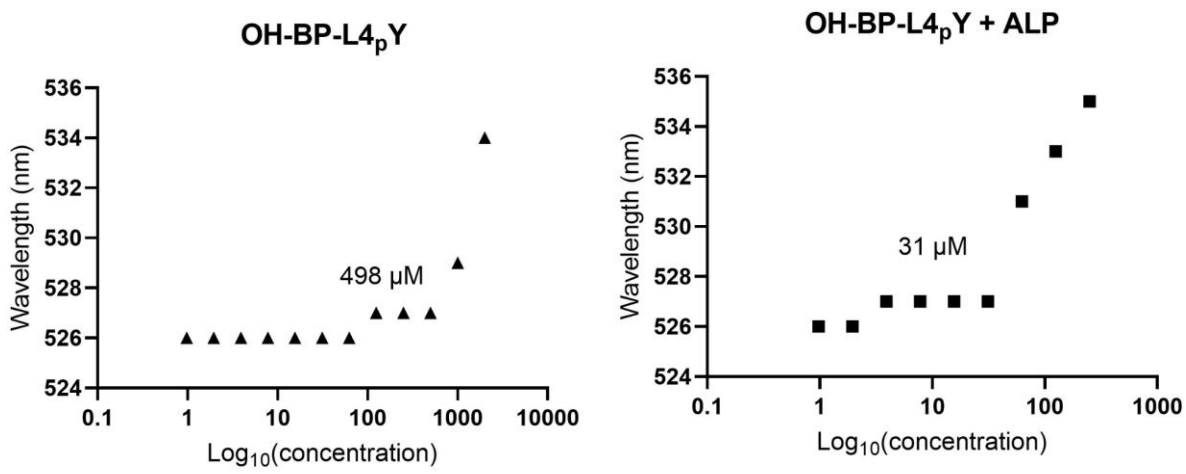


Figure S25. CMC determination of OH-BP-L4$_p$Y in the absence or presence of ALP (0.1 U/mL) for 48 h in PBS.

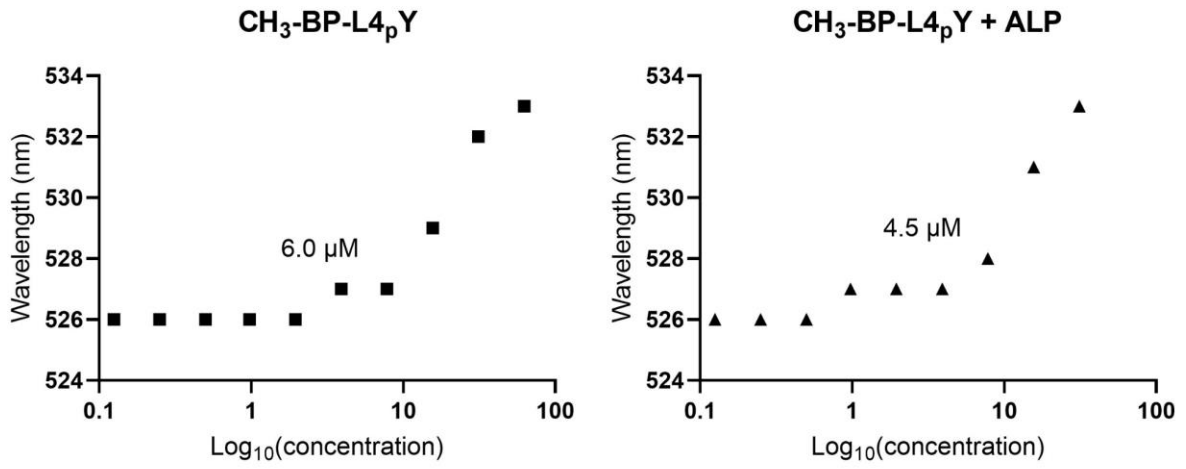


Figure S26. CMC determination of CH_3-BP-L4$_p$Y in the absence or presence of ALP (0.1 U/mL) for 48 h in PBS.

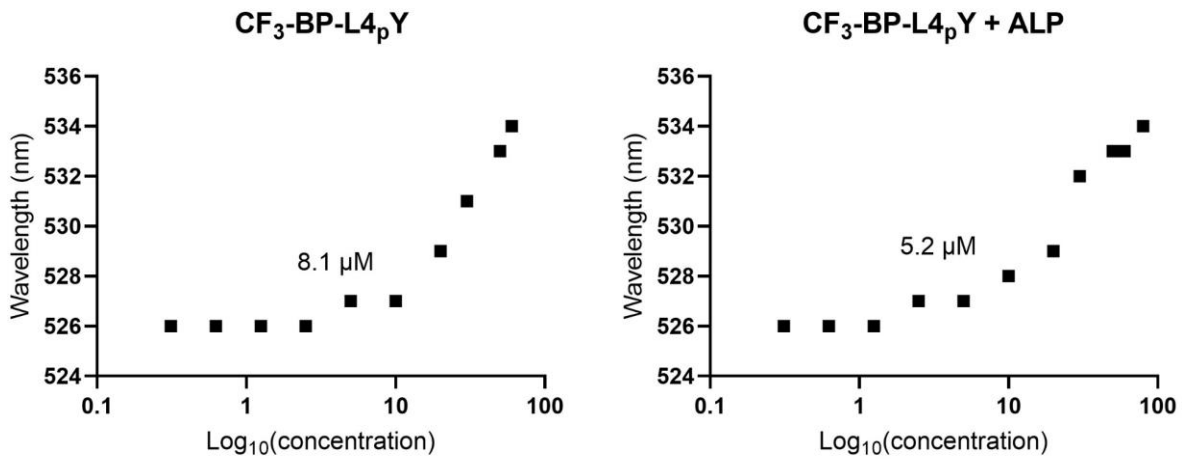


Figure S27. CMC determination of CF_3-BP-L4$_p$Y in the absence or presence of ALP (0.1 U/mL) for 48 h in PBS.

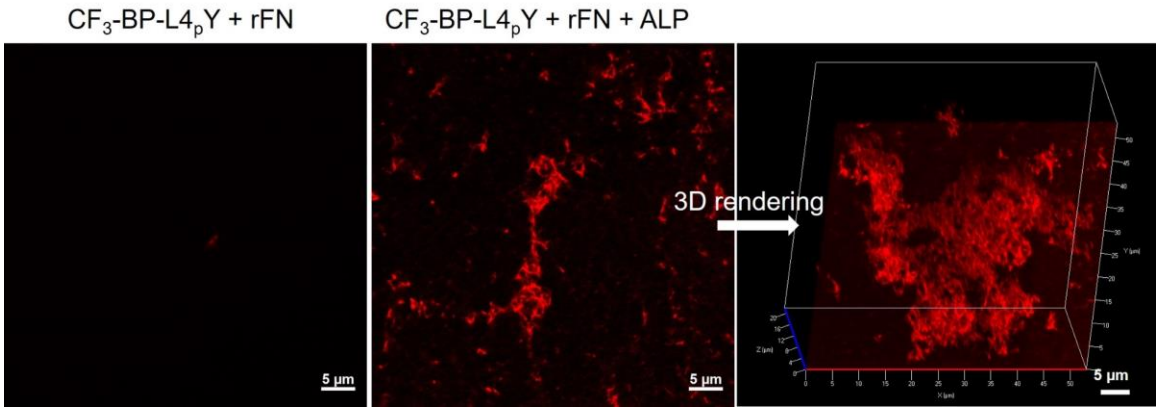


Figure S28. Confocal images of CF3-BP-L4$_p$Y (50 μM) incubated with rFN (50 μg/mL) in the presence or absence of ALP (0.1 U/mL).

Supporting References

1. J. Guo, S. T. Rich-New, C. Liu, Y. Huang, W. Tan, H. He, M. Yi, X. Zhang, E. H. Egelman and F. Wang, *Chem*, 2023, **9**, 2530-2546.