Bio-inspired assembly in phospholipid bilayer： Effective regulation of electrostatic and hydrophobic interaction for plasma membrane targeted probes

Lei Shi, Kun Li,* Yan-Hong Liu, Xin Liu, Qian Zhou, Qi Xu, Shan-Yong Chen and Xiao-Qi Yu*
Key Laboratory of Green Chemistry and Technology, Ministry of Education, College of Chemistry, Sichuan University
Chengdu 610064, China
E-mail: kli@scu.edu.cn, xqyu@scu.edu.cn

ABSTRACT: Recently, supramolecular assembly experiences rapid development. However, the assembly in bio-system with multiforces still needs to be explored. Herein, inspired by the natural property of phospholipid bilayer (PB), three probes which could assemble with phospholipid bilayer through hydrophobic and electrostatic interaction are reported for rapid and accurate specific imaging of plasma membrane. Besides, the hydrophobic interaction is demonstrated as the main force to assemble with PB through simulated vesicles experiments. What' more, we have captured the picture of probes assembly with the phospholipid bilayer in the cell at the first time, to best of our knowledge. Moreover, the electrostatic interaction is also demonstrated to reduce assemble period and increasing assemble continuity and retention. Furthermore, the good assemble specificity and biocompatibility endow the probes could work well in more complex 3D cell model.

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1.Experimental Procedures

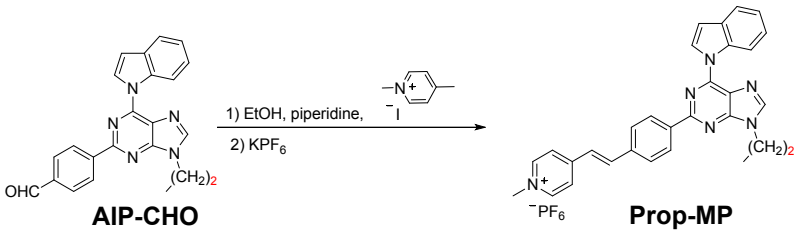
1.1 General information

All chemicals and solvents were commercially available and were used without further purification. 2,6-dichloropurine, 1-bromopropane, 1-bromopentane, indole, 4-formylphenylboronic acid, ethyl sulfonic acid sodium, butyl sulfonic acid sodium, octyl sulfonic acid sodium, dodecyl sulfonic acid sodium, positive charged cetyl trimethyl ammonium bromide and 1,2-dioleoyl-sn-glycero-4-phosphocholine were purchased from Innochem. B16 cells, HEK293 cells and HepG2 cells were purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. DiO, MTS assay, 3D cell vability assay, luminescent cell viability assay and Live/Dead cell imaging kit were bought from Thermo Fisher Scientific. Tissue slices were bought from Lilai Biotechnology. 4-(6-(1H-indol-1-yl)-9-propyl-9H-purin-2-yl) benzaldehyde (**AIP-CHO**)[1] and trimethylammoniopropyl)-4-methylpyridinium dibromide[2] were synthesized according to the literature methods.

^{1}H NMR, ^{13}C NMR spectra were measured on a Bruker AM400 NMR spectrometer. Proton Chemical shifts of NMR spectra were given in ppm relative to internals reference TMS (0.00 ppm). ESI-HRMS spectral data were recorded on a Finnigan LCQDECA mass spectrometer. Fluorescence emission spectra were obtained using Hitachi F-7000 spectrometer at 298 K. Absorption spectra were recorded on a Hitachi UV-1900 UV-Visible Spectrophotometer. The absolute fluorescence quantum yields were collected on a Horiba Fluorolog-3 fluorescence spectrometer with a calibrated integrating sphere system. The fluorescence lifetime was measured using a Hamamatsu Compact Fluorescence Lifetime Spectrometer C11367. All the calculations were performed using Gaussian 09 package. MTS method and 3D cell viability assay were used for testing the cell viability and described in the experimental section. All cells were obtained from Shanghai Institute of Biochemistry and Cell Biochemistry and Cell Biology, Chinese Academy of Science. Confocal lasing scanning microscopic (CLSM) images of single-photo were obtained using LSM 780 (Zeiss). Unless otherwise noted, materials were obtained from commercial suppliers and were used without further purification. All the solvents were dried according to the standard methods prior to use. All of the solvents were either HPLC or spectroscopic grade in the optical spectroscopic studies.

1.2 Reaction procedures

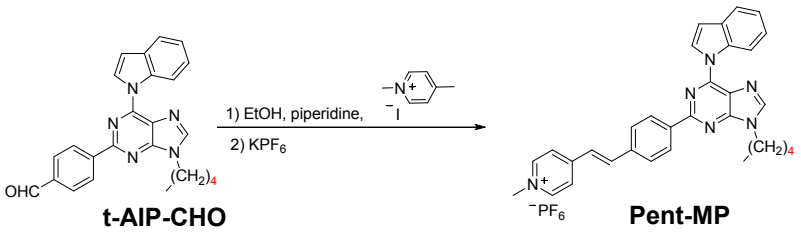
4-(6-(1H-indol-1-yl)-9-propyl-9H-purin-2-yl) benzaldehyde (**t-AIP-CHO**) was synthesized according to the literature method[1] by changing the 1-bromopropane to 1-bromopentane.



(E)-4-(4-(6-(1H-indol-1-yl)-9-propyl-9H-purin-2-yl) styryl)-1-methylpyridin-1-ium hexafluorophosphate(V) (**Prop-MP**)

After **AIP-CHO** (381 mg, 1 mmol) and 1,4-dimethylpyridin-1-ium iodide (235 mg, 1 mmol) was added in EtOH (10 mL), the piperidine (0.05 mL) was dropped to the stirred solution. Then the mixture was stirred at room temperature for about 12 h. After the reaction was completed based on the TLC, removed the solvent and then dissolved in acetone (10 mL) with saturated potassium hexafluorophosphate solution (10 mL). After stirring at room temperature for 2 hour, the acetone was removed under reduced pressure and then crude product was got. The crude product was purified by column chromatography on silica gel. Elution with MeOH/DCM=20/1 gave **Prop-MP** as a deep-yellow solid in 25% yield.

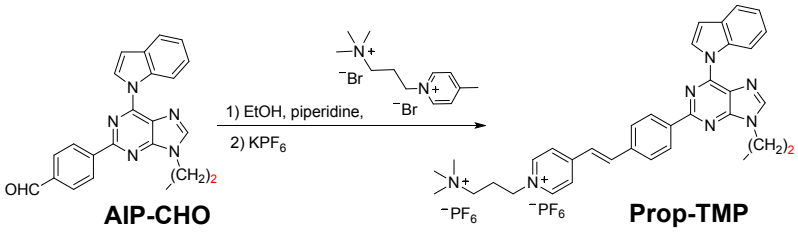
^{1}H NMR (400 MHz, DMSO-d_6)δ 9.23-9.13 (d, 1H), 9.07-9.03 (d, 1H), 8.89-8.85 (d, 2H), 8.70-8.68 (s, 1H), 8.62-8.58 (d, 2H), 8.26-8.23 (d, 2H), 8.11-8.05 (d, 1H), 7.99-7.95 (d, 2H), 7.74-7.70 (d, 1H), 7.66-7.61 (d, 1H), 7.47-7.42 (t, 1H), 7.34-7.29 (t, 1H), 6.95-6.93 (d, 1H), 4.40-4.34 (t, 2H), 4.28-4.24 (s, 3H), 1.98-1.93 (t, 2H), 0.89-0.84 (t, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 157.0, 154.0, 152.6, 149.0, 146.1, 145.6, 140.2, 139.6, 137.4, 135.6, 130.6, 129.0, 128.9, 128.8, 124.8, 124.3, 124.1, 123.2, 121.5, 121.2, 116.7, 108.5, 47.4, 43.7, 29.2, 28.6, 22.0, 14.2. HRMS (ESI): *m/z*: Calcd for $C_{30}H_{27}N_6^+$: 471.2292 ; $[M\text{-}PF_6]^+$ Found: 471.2284.



(E)-4-(4-(6-(1H-indol-1-yl)-9-pentyl-9H-purin-2-yl) styryl)-1-methylpyridin-1-ium hexafluorophosphate(V) (**Pent-MP**)

After **t-AIP-CHO** (409 mg, 1 mmol) and 1,4-dimethylpyridin-1-ium iodide (235 mg, 1 mmol) was added in EtOH (10 mL), the piperidine (0.05 mL) was dropped to the stirred solution. Then the mixture was stirred at room temperature for about 12 h. After the reaction was completed based on the TLC, removed the solvent and then dissolved in acetone (10 mL) with saturated potassium hexafluorophosphate solution (10 mL). After stirring at room temperature for 2 hour, the acetone was removed under reduced

pressure and then crude product was got. The crude product was purified by column chromatography on silica gel. Elution with MeOH/DCM=20/1 gave **Pent-MP** as a yellow solid in 37% yield.
^{1}H NMR (400 MHz, DMSO-d_6) δ 9.22-9.20 (d, 1H), 9.06-9.03 (d, 1H), 8.88-8.84 (d, 2H), 8.67-8.65 (s, 1H), 8.59-8.52 (d, 2H), 8.25-8.21 (d, 2H), 8.09-8.02 (d, 1H), 7.96-7.91 (d, 2H), 7.73-7.69 (d, 1H), 7.64-7.57 (d, 1H), 7.47-7.41 (t, 1H), 7.33-7.28 (t, 1H), 6.94-6.92 (d, 1H), 4.36-4.30 (t, 2H), 4.27-4.24 (s, 3H), 1.99-1.90 (t, 2H), 1.38-1.27 (m, 4H), 0.89-0.83 (t, 3H).^{13}C NMR (101 MHz, DMSO-d_6) δ 157.0, 154.0, 152.6, 149.0, 146.1, 145.6, 140.2, 139.5, 137.3, 135.6, 130.6, 129.0, 128.8, 124.8, 124.3, 124.1, 123.2, 121.5, 121.2, 116.7, 108.5, 47.4, 43.7, 29.2, 28.6, 22.0, 14.2.HRMS (ESI): *m/z*: Calcd for $C_{32}H_{31}N_6^+$: 499.2605; $[M\text{-}PF_6]^+$ Found: 499.2605.



(E)-4-(4-(6-(1H-indol-1-yl)-9-propyl-9H-purin-2-yl) styryl)-1-(3-(trimethylammonio) propyl) pyridin-1-ium hexafluorophosphate(V) (**Prop-TMP**)
After **AIP-CHO** (381 mg, 1 mmol) and 4-methyl-1-(3-(trimethylammonio)propyl)pyridin-1-ium bromide (352 mg, 1 mmol) was added in EtOH (10 mL), the piperidine (0.05 mL) was dropped to the stirred solution. Then the mixture was stirred at room temperature for about 12 h. After the reaction was completed based on the TLC, removed the solvent and then dissolved in acetone (10 mL) with saturated potassium hexafluorophosphate solution (10 mL). After stirring at room temperature for 2 hour, the acetone was removed under reduced pressure and then crude product was got. The crude product was purified by column chromatography on neutral aluminum oxide. Elution with MeOH/DCM=20/1 gave **Prop-TMP** as a brown solid in 33% yield.
^{1}H NMR (400 MHz, DMSO-d_6) δ 9.23-9.20 (d, 1H), 9.09-9.03 (m, 3H), 8.71-8.69 (s, 1H), 8.62-8.57 (d, 2H), 8.37-8.33 (d, 2H), 8.20-8.14 (d, 1H), 8.02-7.98 (d, 2H), 7.74-7.67 (m, 2H), 7.47-7.42 (t, 1H), 7.34-7.28 (t, 1H), 6.95-6.93 (d, 1H), 4.65-4.60 (t, 2H), 4.40-4.34 (t, 2H), 3.11-3.7 (s, 9H), 2.38-2.48 (m, 4H), 1.99-1.92 (m, 2H), 0.89-0.83 (t, 3H).^{13}C NMR (101 MHz, DMSO-d_6) δ 157.0, 154.1, 153.5, 149.0, 146.2, 145.0, 140.8, 139.7, 137.3, 135.6, 130.6, 129.1, 129.0, 128.9, 124.8, 124.5, 124.3, 123.2, 121.5, 121.2, 116.7, 109.9, 108.6, 62.2, 57.2, 52.9, 45.5, 24.4, 23.0, 11.5. HRMS (ESI): *m/z*: Calcd for $C_{35}H_{39}F_6N_7P^+$: 702.2903; $[M\text{-}PF_6]^+$ Found: 702.2902.

1.3 Preparation of lipid vesicles
The lipid vesicles were prepared according to the processes reported in the literature[3]. Generally, 2.2 mM pure ethyl sulfonic acid sodium, butyl sulfonic acid sodium, octyl sulfonic acid sodium, dodecyl sulfonic acid sodium, positive charged cetyl trimethyl ammonium bromide and 1,2-dioleoyl-sn-glycero-4-phosphocholine were added in PBS, respectively. And the vesicles were shocked in the temperature incubator at 37℃ for 30 min, then sonicated for 1h. After that, the final vesicles were obtained by extruding 10 times through 100 nm pore size polycarbonate filter at 50℃ on a pre-warmed lipid extruder.

1.4 Cell culture
B16 cells were cultured in RPMI Medium 1640 basic (1X) containing 10% fetal bovine serum and 1% Antibiotic–antimycotic at 37℃ in a 5% CO_2/95% air incubator.
HepG2, HEK293 were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum and 1% Antibiotic–antimycotic at 37℃ in a 5% CO_2/95% air incubator.

The 3D cells model of B16 cells were prepared according to the processes reported in the literature[4]. Generally, cell suspensions were diluted in medium containing 0.24% (w/v) methylcellulose at a density of around 1 × 10^6 cells per mL. Then, 40 μL of diluted cells was dropped onto the lids of 10 cm cell culture plates. After 24 h, the spheroids were formed and transferred to agarose-coated 96-well plates. The spheroids were incubated for another 48 h before stained with probes.

1.5 Preparation of the TEM experiments
The TEM experiment was contracted to the Lilai Biotechnology. Generally, the B16 cells were treated with pancreatin, then scattered in the RPMI Medium 1640. After that, the cells were collected in 1.5 mL centrifuge tube with 1500 rpm for 10 min. The cells were scattered in PBS (Blank group) and 5 μM Prop-TMP solution (Sample group) for 1 min, and then centrifuged with 1500 rpm for 10 min again. Then discarded the supernatant, and slowly added about 0.5% glutaraldehyde fixation solution along the tube wall (PBS basic), then stored the cells at 4℃ for 10 minutes. Next, after further centrifuging at 10000 rpm for 15 min, the supernatant was discarded, and slowly added about 3% glutaraldehyde fixation solution along the tube wall (PBS basic), then stored the cells at 4℃. The fixed sample was given to Lilai Biotechnology to do the following process, to note, the volume of fixed sample should be larger than half a mung bean.

1.6 Cell imaging
2D cells were grown on a cover slip overnight in a 35-mm petri dish. The cells were stained with certain dye at certain concentration for certain time (by adding 2 μL of stock solution in DMSO to a 1 mL of culture medium with DMSO < 0.1 vol %). The cells were imaged under CLSM using proper excitation and emission filters for each dye: λ_{ex}=405 nm, λ_{em}=420-600 nm.

3D B16 cell spheroids were transferred from agarose-coated 96-well plates to a new 96-well plate. After adding 100 μL RPMI Medium 1640 basic containing 10 μM probe per well and staining for 90 minutes, the 3D cells were imaged under CLSM directly. λ_{ex}=405 nm, λ_{em}=420-600 nm.

1.7 Co-localization imaging

2D cells were grown on a cover slip overnight in a 35-mm petri dish. The cells were stained with 5 μM DiO for10 minutes, then washed with 1 mL PBS three times. After that, the cells were stained with 5 μM different probes for 1 minute, and then used for imaging directly. For compounds, λ_{ex}=405 nm, λ_{em}=420-480 nm; for DiO, λ_{ex}=488 nm, λ_{em}=530-560 nm.

3D B16 cell spheroids were transferred from agarose-coated 96-well plates to a new 96-well plate. After adding 100 μL RPMI Medium 1640 basic containing 10 μM Prop-TMP and 2μM DiO per well and staining for 90 minutes, the 3D cells were imaged under CLSM directly. For Prop-TMP, λ_{ex}=405 nm, λ_{em}=420-480 nm; for DiO, λ_{ex}=488 nm, λ_{em}=530-560 nm.

1.8 Cytotoxicity study

Toxicity toward 2D B16 cells was determined by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) reduction assay following literature procedures. About 10000 cells per well were seeded in 96-well plates and cultured overnight for 70-80% cell confluence. The medium was replaced with 100 μL of fresh medium with different concentration of probes, to which 100 μL complexes at 200 μL. 24 hours later, 100 μL of 20% MTS solution in RPMI Medium 1640 basic was replaced with the old medium in each well for additional 0.5h incubation. The metabolic activity of the probes treated cells was expressed as a relative to untreated cell controls taken as 100% metabolic activity.

And the toxicity toward 3D B16 cells was determined by CellTiter-Glo 3D Cell Viability Assay. According to the 3D cell culture mentioned before, the stale 1640 medium was replaced with 100 μL fresh one containing different concentration of probes. 24 hours later, the 3D cell spheroids were transferred to new opaque 96-well plates. Then the medium was replaced with a mixture containing 100 μL 1640 medium and 100 μL kit. 25 minutes later, the plates were used for chemiluminescent detection directly.

For the imaging of the toxicity toward 3D B16 cells, the Live/Dead cell imaging kit was adopted. The 3D cell spheroids were incubated with different concentration of probes for 24 hours. Then the medium was replaced by a mixture containing 100 μL fresh 1640 medium and 100 μL kit (a mixture of FITC and Texas Red). 15 minutes later, the 3D cell spheroids were used for CLSM imaging directly. For compounds,λ_{ex}=405 nm, λ_{em}=420-480 nm; for FITC, λ_{ex}=488 nm, λ_{em}=505-550 nm; for Texas Red, λ_{ex}=570 nm, λ_{em}=585-620 nm.

2. Molar Extinction Coefficient of all compounds

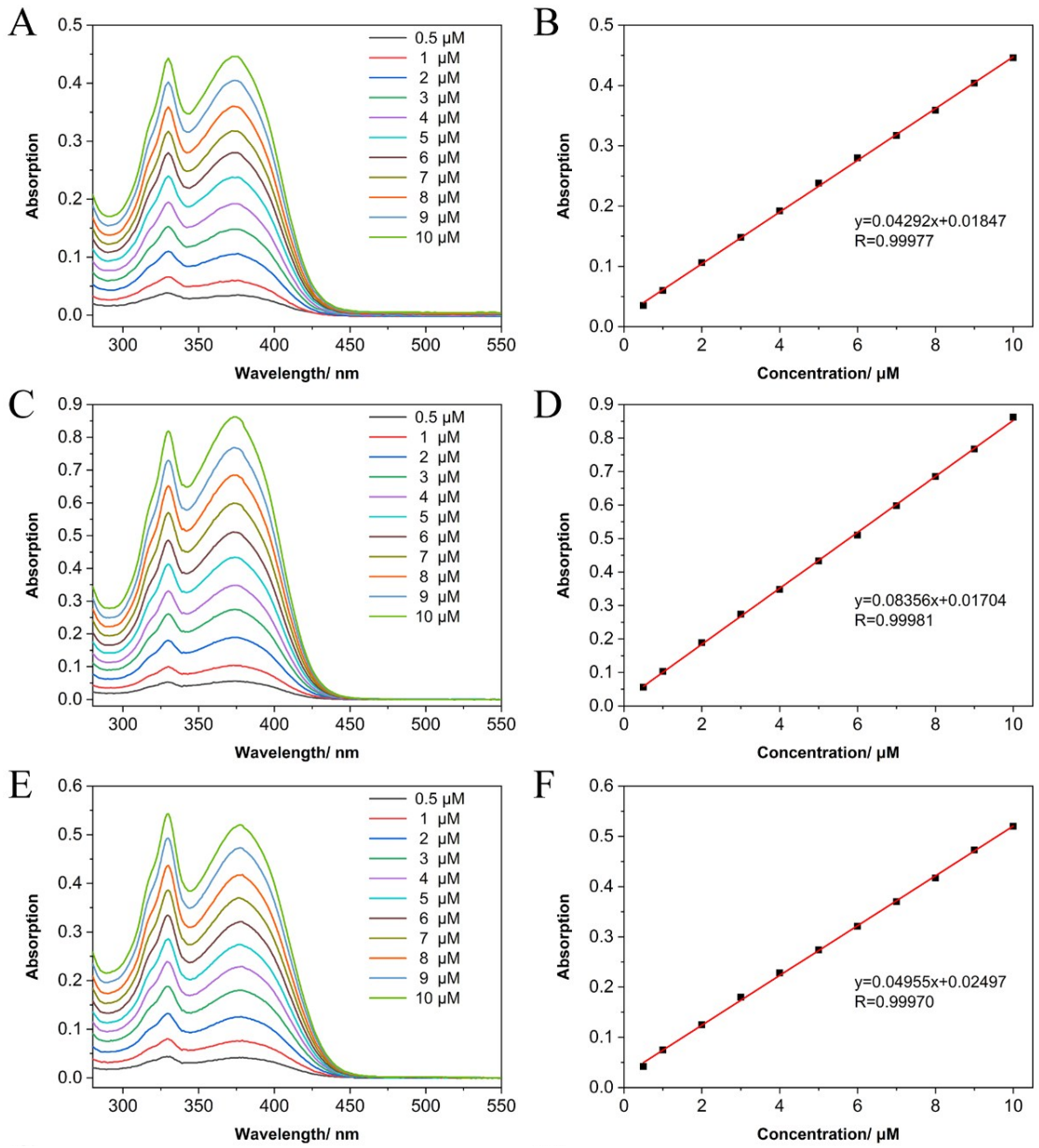


Figure S1 UV spectra of compound A) Prop-MP C) Pent-MP E) Prop-TMP at different concentrations (0.5, 1, 2, 3, 5, 6, 8, 10 μM); Absorption-concentration curve of compound B) Prop-MP D) Pent-MP F) Prop-TMP.

Table S1. Summary of all the compounds' molar extinction coefficient.

Compounds	λ_{Abs} (nm)	ε(M^{-1} cm^{-1})
Prop-MP	372	4.30×10^4
Pent-MP	374	8.36×10^4
Prop-TMP	377	4.96×10^4

3. Fluorescence spectra of all compounds in DMSO/TL mixtures

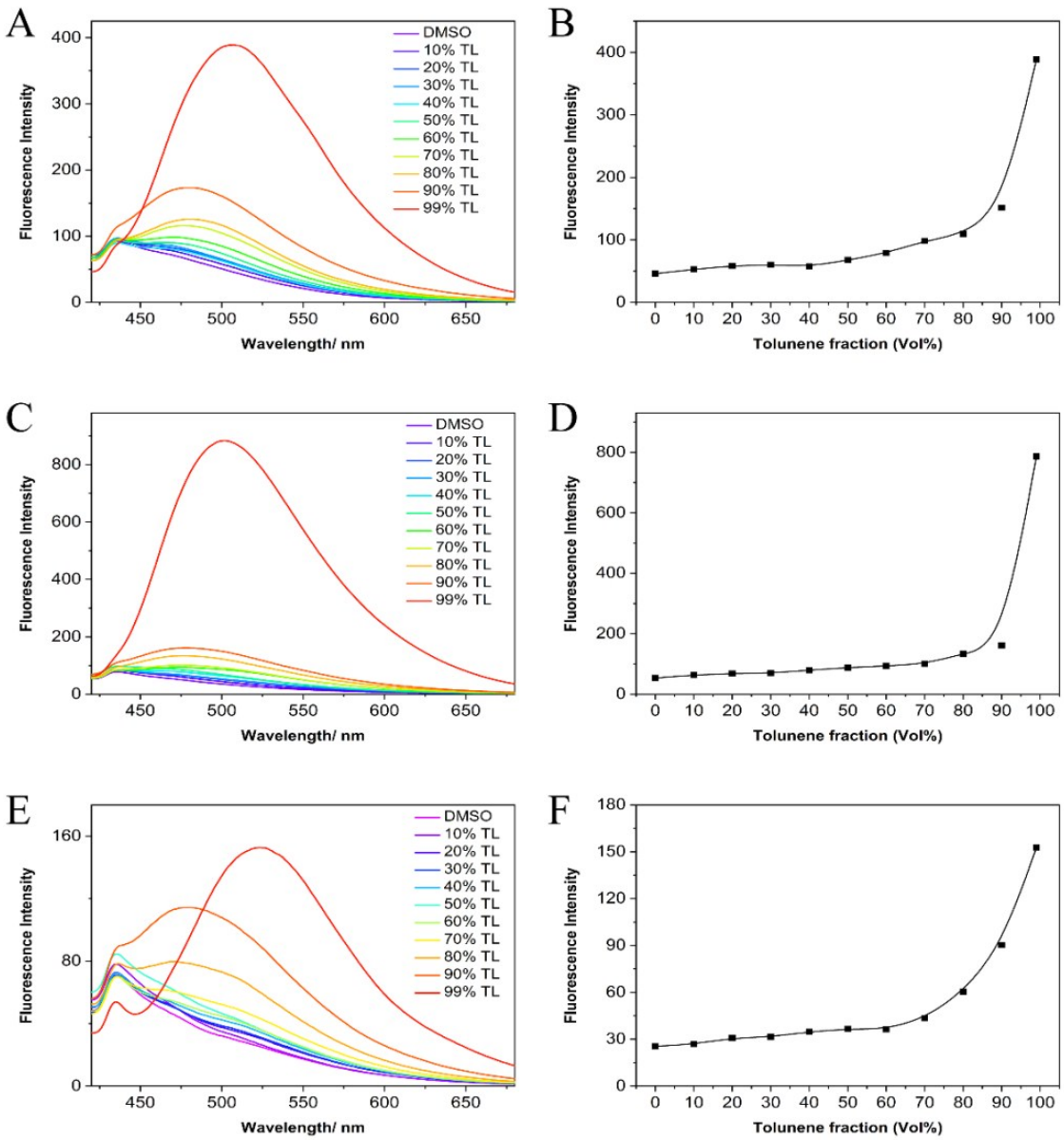


Figure S2 Fluorescence spectra of （A) Prop-MP, （C) Pent-MP, （E) Prop-TMP in DMSO/TL mixtures and dependence of the I/I_0 ratios of （B) Prop-MP, （D) Pent-MP, （F) Prop-TMP on the solvent composition of the DMSO/TL mixture. Concentration: 5 μM, λ_{ex} = 370 nm..

4. Fluorescent lifetime and quantum yield of all compounds in solution, aggregation and in solid

Table S2. Quantum yield of all compounds in DMSO, TL and in solid state.

Compound	Quantum yield in solution (%)	Quantum yield in aggregation (%)	Quantum yield in solid state (%)
Prop-MP	＜0.1	9.49	7.9
Pent-MP	＜0.1	8.54	12.2
Prop-TMP	＜0.1	6.18	5.1

Table S3. Optical properties of all compounds

Compound	λ_{abs}[a]	ε[a]	λ_{em} (nm)			τ_{avg}
	(nm)	($M^{-1}cm^{-1}$)	Soln(Φ_F)[b]	Aggr(Φ_F)[c]	Solid(Φ_F)[d]	(s)[e]
Prop-MP	372	4.30×10^4	n.d. (<0.1)	507 (9.5%)	527 (7.9)	2.40×10^{-1}
Pent-MP	374	8.36×10^4	n.d. (<0.1)	502(8.5%)	532 (12.2)	1.26×10^{-4}
Prop-TMP	377	4.96×10^4	n.d. (<0.1)	524 (6.2)	530 (5.1)	1.25×10^{-4}

[a] Absorption maximum in DMSO; [b] Emission maximum of solution state in DMSO; [c] Emission maximum of aggregation state in tolunene; [d] Emission maximum in solid state; [e] Solid-state fluorescence lifetime, measured in ambient conditions. Fluorescence quantum yield determined by a calibrated integrating sphere.

5. Assemble with different simulated lipid vesicles

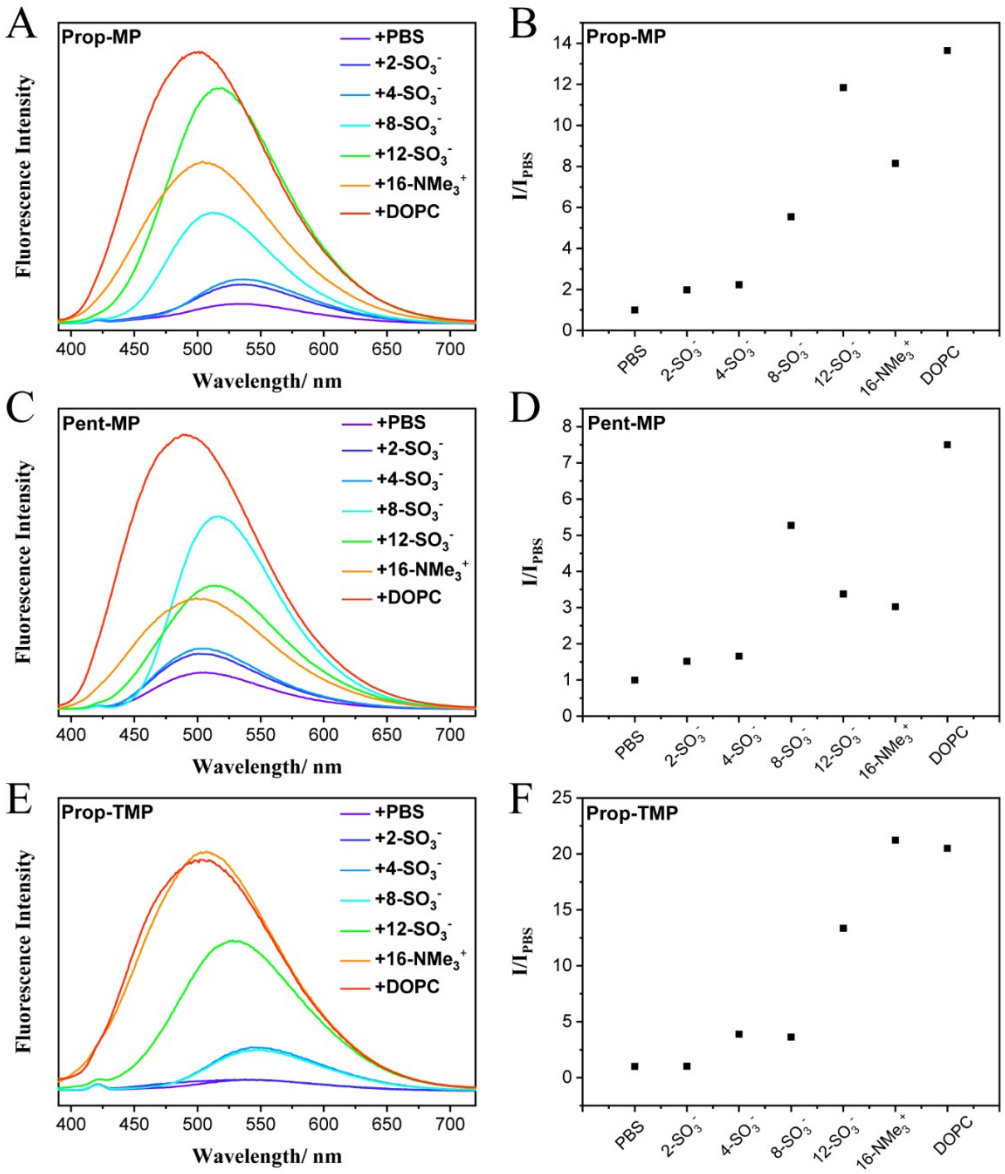


Figure S3 The fluorescent spectrum of 5 μM (A) Prop-MP (C) Pent-MP (E) Prop-TMP assembled with different pure vesicles; The fluorescent intensification factor of (B) Prop-MP (D) Pent-MP (F) Prop-TMP assembled with different pure vesicles compared with in PBS

After vesicles preparation, the probes were mixed with them. For example, 5 μmol **Prop-TMP** were mixed with PBS, 2-SO_3^- vesicle, 4-SO_3^- vesicle, 8-SO_3^- vesicle, 12-SO_3^- vesicle, 16-NMe_3^+ vesicle and DOPC vesicle, respectively.
As shown in Figure S3, the probes were nearly nonfluorescent in PBS, however, with the increasing of the vesicles' alkyl length, the fluorescence was significantly blue shifted and enhanced. This phenomenon may attribute to the assemble induced RIM process, that the short alkyl group of vesicles could not suppress the rotation and vibration effectively thus consumed the absorbed excitation energy via nonradiative decay when the probes assembled with vesicles.
Interestingly, we found that, the positive charged probes could not only assemble with negative charged vesicles (2-SO_3^- vesicle, 4-SO_3^- vesicle, 8-SO_3^- vesicle, 12-SO_3^- vesicle), but also form a stable assembly with the positive charged vesicle (16-NMe_3^+ vesicle) and naturel charged vesicle (DOPC vesicle). This phenomenon didn't strictly observe electrostatic interaction, representing the hydrophobic interaction overcame the charge repulsion of vesicles with same polarity and was the main force of assemble.

6. Transmission electron microscope experiment of Prop-TMP and cells

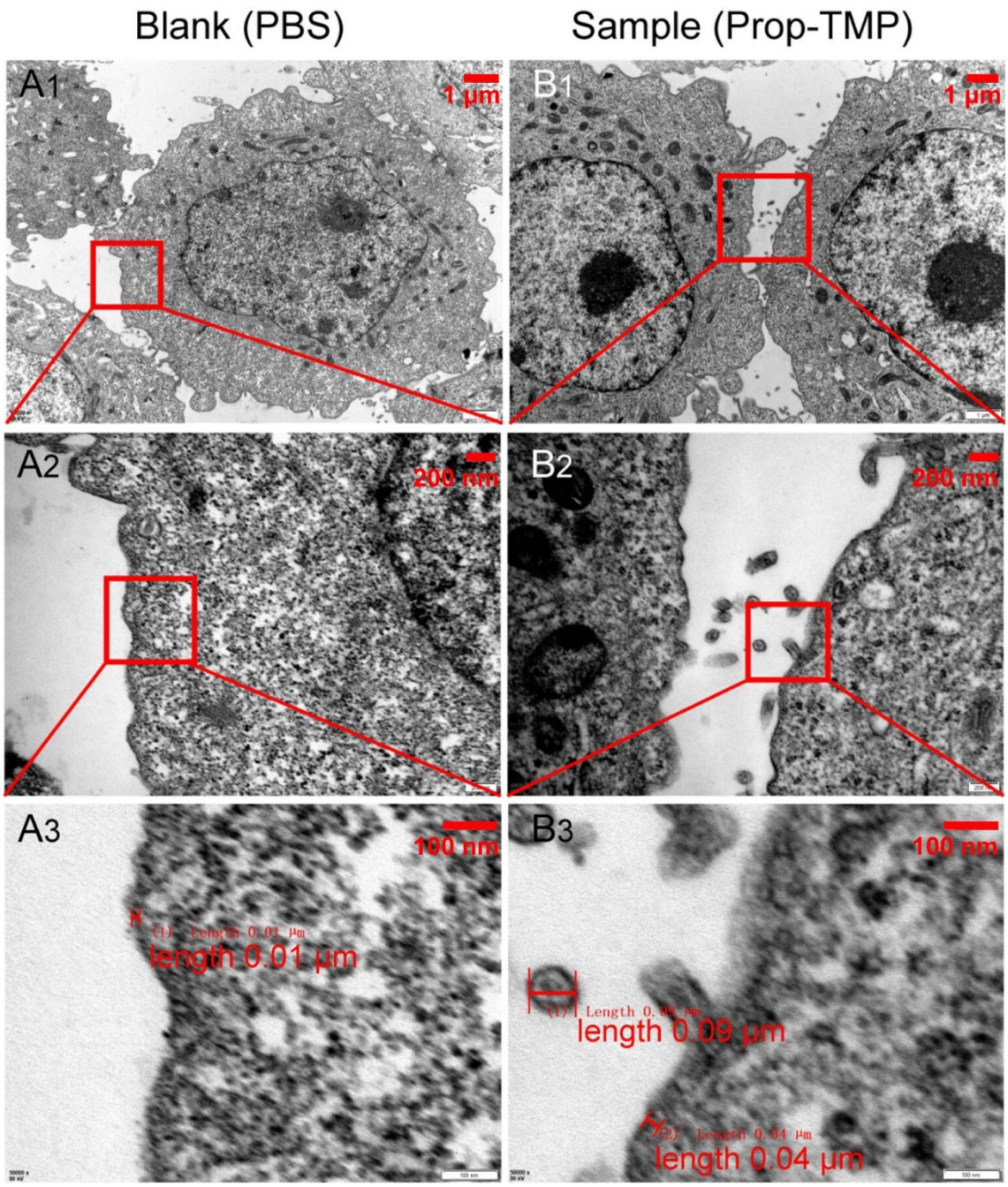


Figure S4 TEM images of cells incubated in (A) PBS and (B) 5 μM Prop-TMP for 1 minutes; figure 2 is the zooming in version of figure 1, figure 3 is the zooming in version of figure 2.

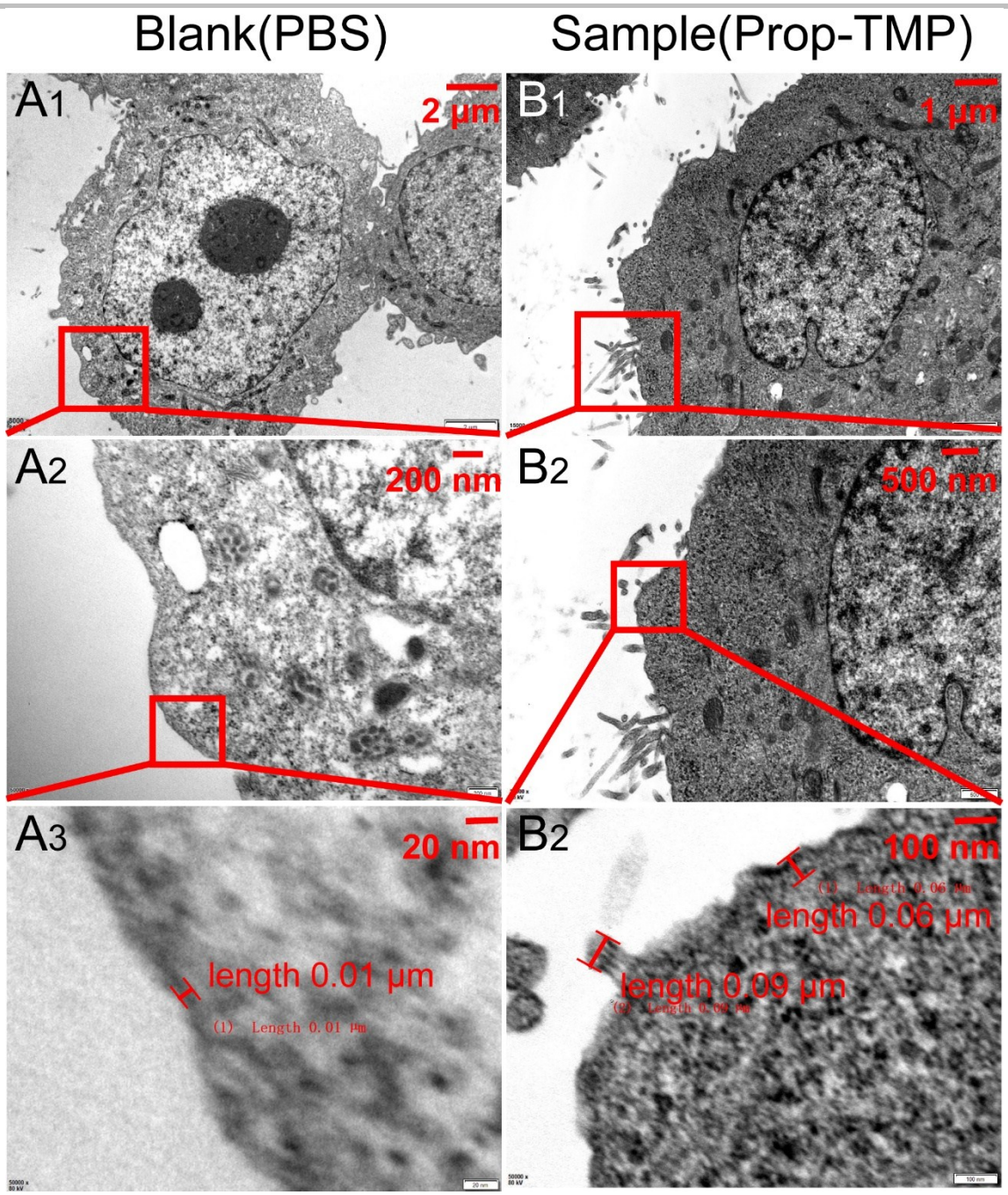


Figure S5 TEM images of cells incubated in (A) PBS and (B) 5 μM Prop-TMP for 1 minutes; figure 2 is the zooming in version of figure 1, figure 3 is the zooming in version of figure 2.

7. 2D cell imaging with different categories of cells

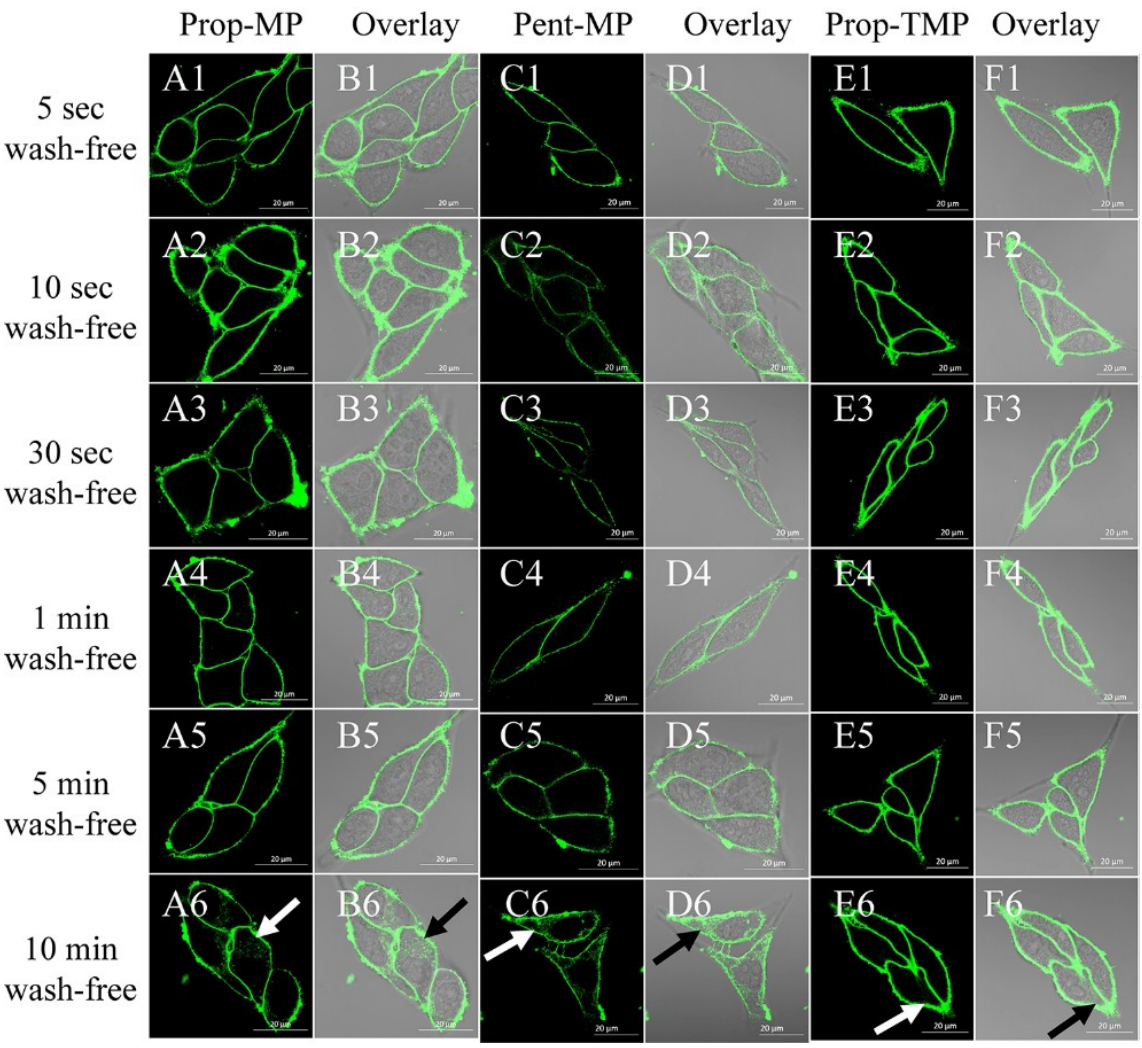


Figure S6 B16 cell stained in different time (shown as left title) with different probes. A) Prop-MP and B) overlay with its bright field; C) Pent-MP and D) overlay with its bright field; E) Prop-TMP and F) overlay with its bright field overlay with its bright field. λ_{ex}=405 nm, λ_{em}=420-600 nm.

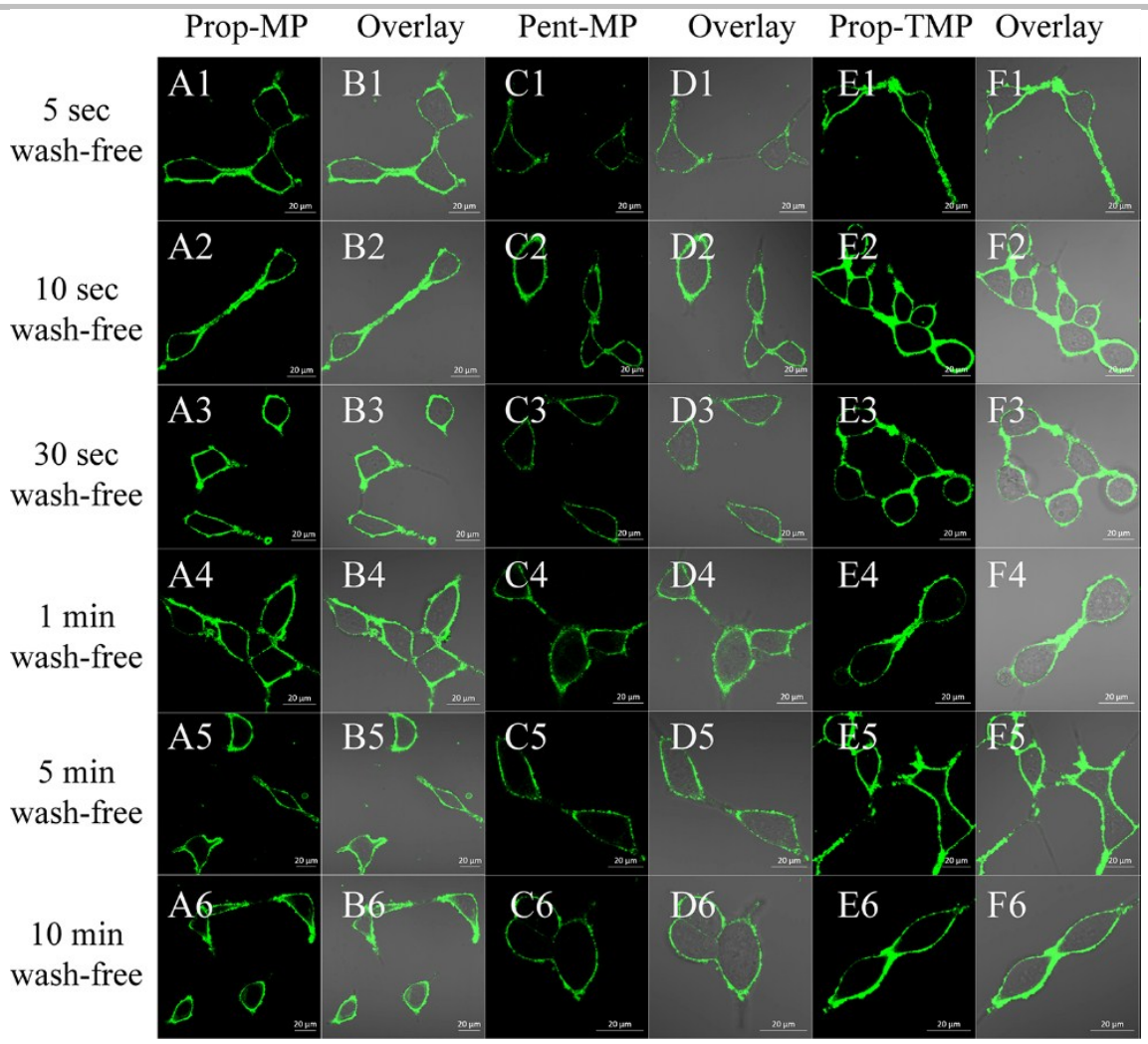


Figure S7 HEK 293 cell stained in different time (shown as left title) with different probes. A) Prop-MP and B) overlay with its bright field; C) Pent-MP and D) overlay with its bright field; E) Prop-TMP and F) overlay with its bright field overlay with its bright field. λ_{ex}=405 nm, λ_{em}=420-600 nm.

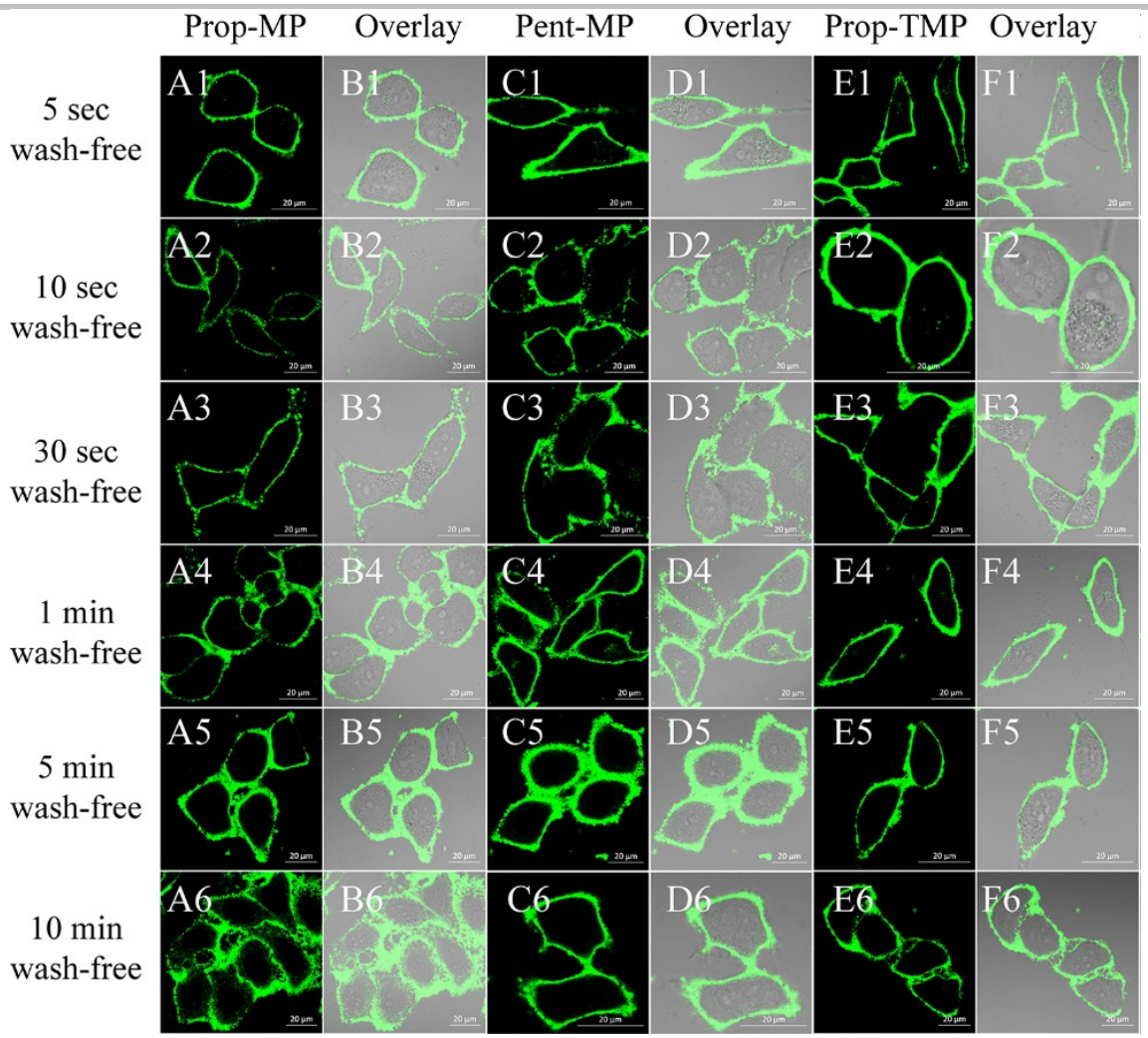
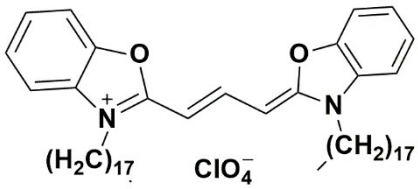


Figure S8 HepG2 cell stained in different time (shown as left title) with different probes. A) Prop-MP and B) overlay with its bright field; C) Pent-MP and D) overlay with its bright field; E) Prop-TMP and F) overlay with its bright field overlay with its bright field. λ_{ex}=405 nm, λ_{em}=420-600 nm.

8. Co-localization experiments of all compounds



DiO

Figure S9 Chemical structure of commercial plasma membrane dye DiO.

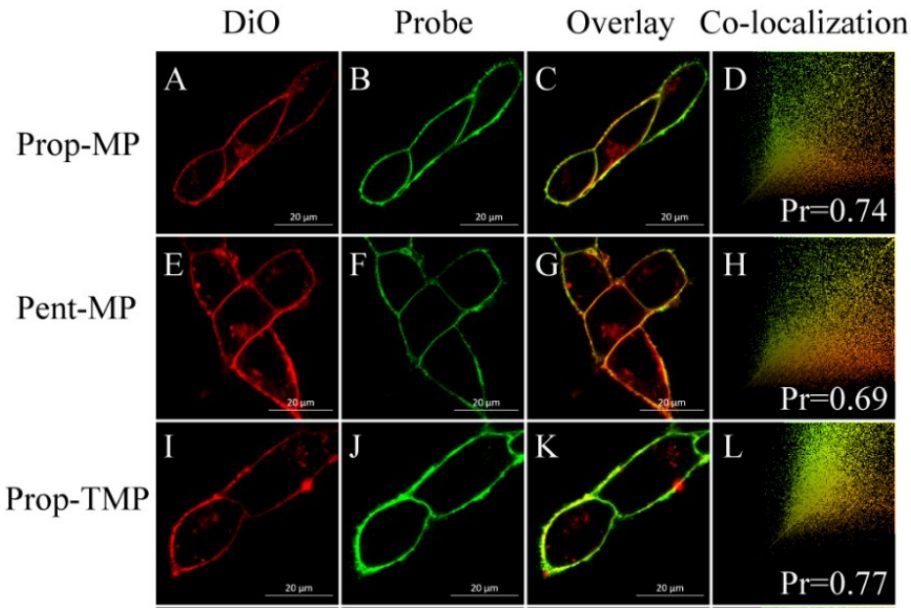


Figure S10 Live B16 cells incubated with DiO and (A-D) Prop-MP, (E-H) Pent-MP, (I-L) Prop-TMP. Red channel of DiO, λ_{ex}=488 nm, λ_{em}=530-560 nm; green channel of probes, λ_{ex}=405 nm, λ_{em}=420-470 nm.

The Pearson correlation coefficient (PCC) of Prop-MP, Pent-MP, and Prop-TMP with DiO was calculated to be 0.74, 0.69 and 0.77, respectively.

9. Photostability of all compounds

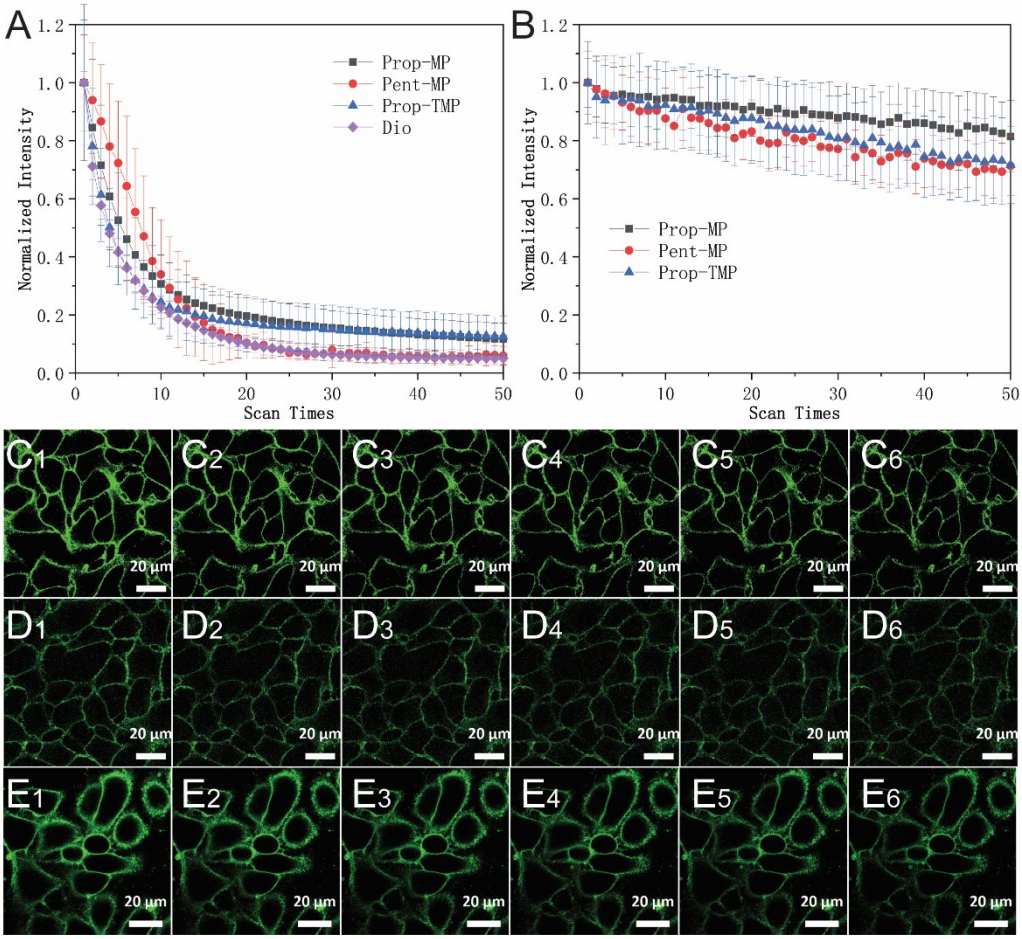


Figure S11 (A) Photostability of all compounds and DiO under the laser power of 100%; (B) Photostability of all compounds under the laser power of 2%; 1-6 represented the pictures of B16 cell stained with (C) Prop-MP, (D) Pent-MP, (E) Prop-TMP after 1, 10, 20, 30, 40, 50 times scanning.

As shown in Figure S9A, our probes and commercial dye DiO were quenched quickly under the laser power at 100%. However, at the power of 2%, our probes could work well in long-term imaging (Figure S9B), and the pictures after 1, 10, 20, 30, 40, 50 times scanning were shown in Figure S8C-E, representing their good photostability at working laser powder (total laser power is 25 mW).

10. 3D B16 cell imaging with all compounds

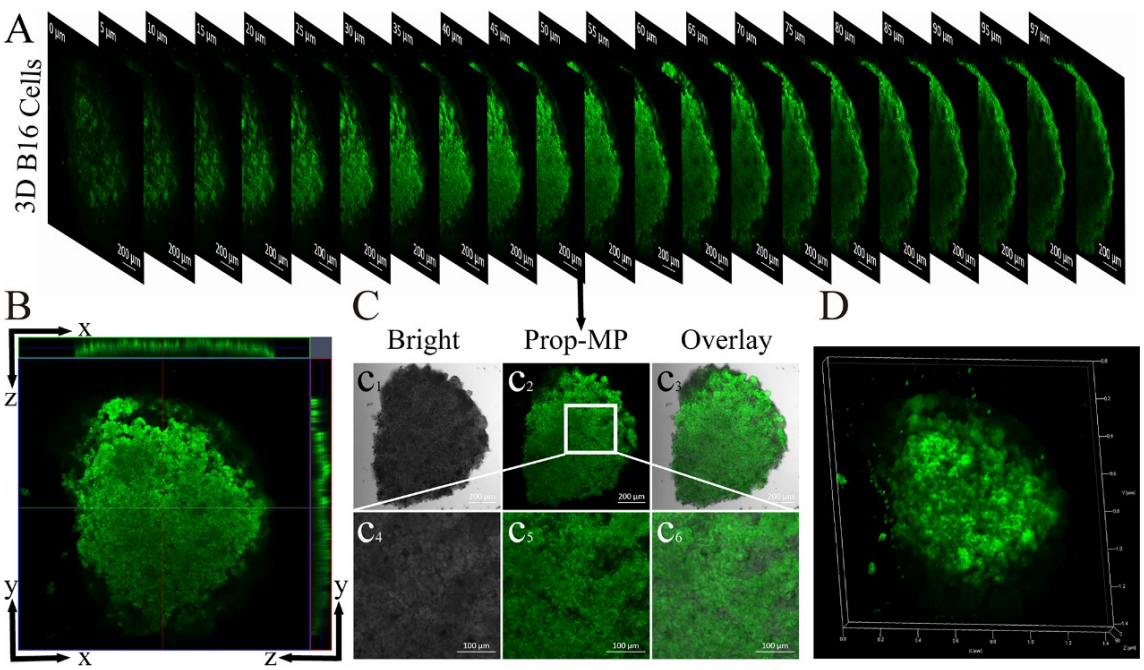


Figure S12 Confocal laser microscopy images of a 3D multicellular spheroid after incubating with **Prop-MP** after 1.5 hours without washing. (A) Z-stack images of the 3D spheroid from 0-120 μm. (B) Cross-sectional images of the x- and y-axis; (C) The Z-axis cut layer at 60 μm; (D)Three-dimensional reconstruction.

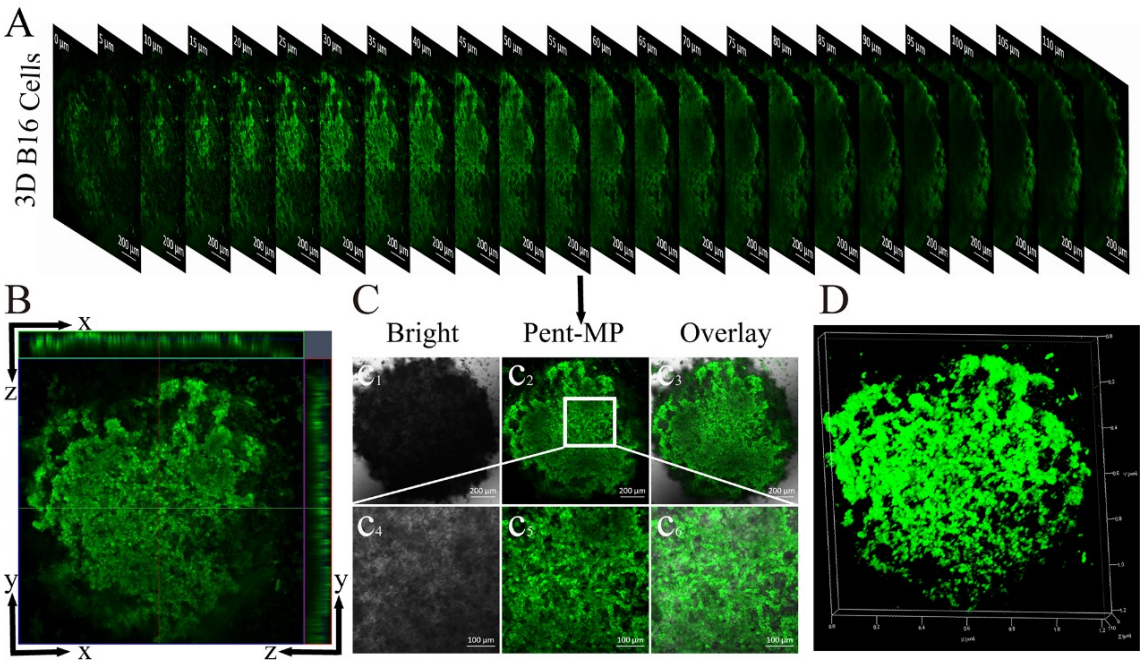


Figure S13 Confocal laser microscopy images of a 3D multicellular spheroid after incubating with **Pent-MP** after 1.5 hours without washing. (A) Z-stack images of the 3D spheroid from 0-120 μm. (B) Cross-sectional images of the x- and y-axis; (C) The Z-axis cut layer at 60 μm; (D)Three-dimensional reconstruction.

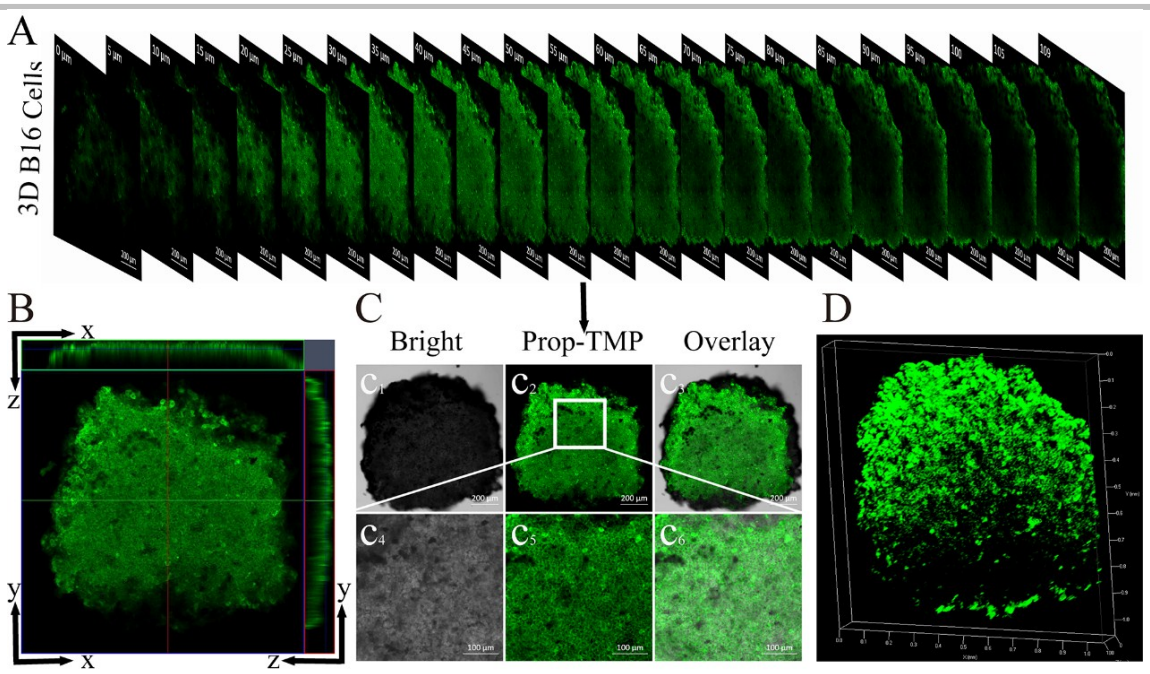


Figure S14 Confocal laser microscopy images of a 3D multicellular spheroid after incubating with **Prop-TMP** after 1.5 hours without washing. (A) Z-stack images of the 3D spheroid from 0-120 μm. (B) Cross-sectional images of the x- and y-axis; (C) The Z-axis cut layer at 60 μm; (D)Three-dimensional reconstruction.

As shown in Figure S14A, the assembling level in different layers of the spheroids after 1.5 h of incubation with **Prop-TMP**, and the Z-stack green fluorescence intensity at the interior layer of the spheroid were observed indicating the good diffusivity this compound had. As shown in Figure S14B, a considerable amount of green fluorescence was observed in all the three sections indicating the reasonable uniformity. As the Z-axis cut layer at 60 microns was shown in Figure S14C, the membranes of honeycombed cell could be clear presented (Figure S14c_2 and S14c_3), and the honeycomb shape of PM could be clearly revealed (Figure S14c4-c6). Besides, Figure S14D showed the assembling of Pent-TMP in the inner and outer layers of the entire 3D cell model as well as the stack of the fluorescence signal in the Z-axis. These results demonstrated **Prop-TMP** could effectively assemble with the PM of the 3D multicellular spheroids and diffuse rapidly equably.

11. Cytotoxicity of all compounds on B16 cells in 2D/3D cell model

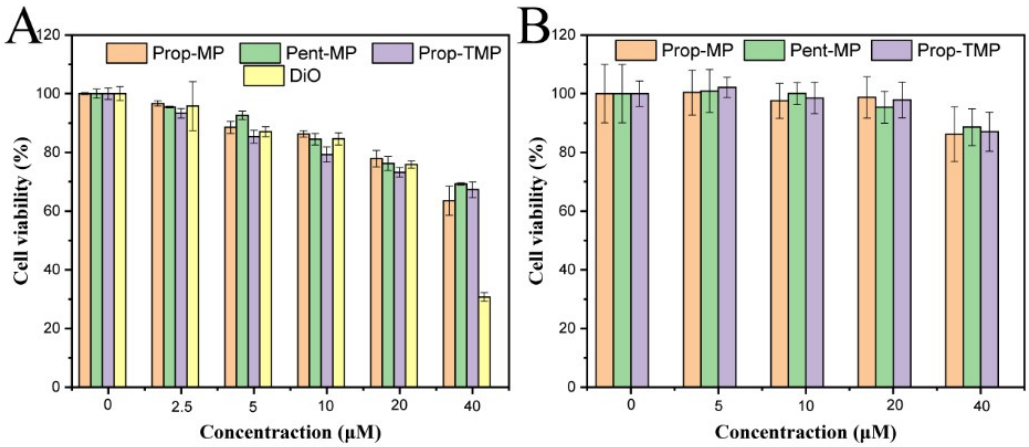


Figure S15 Cell viabilities of A) 2D B) 3D B16 cells after incubation with different concentrations of probes (5, 10, 20, 40 μM) for 24 h.

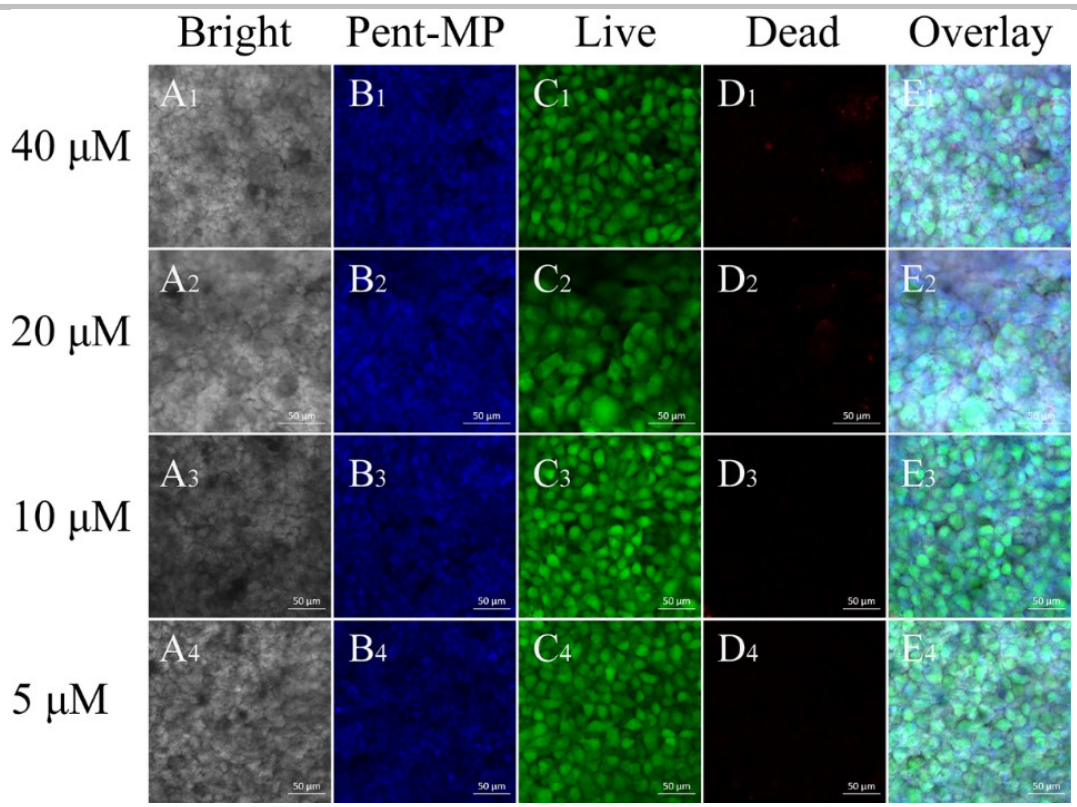


Figure S16 Cell viability of 3D B16 cells treated with different concentration of **Prop-MP** assessed by FITC (Live) and Texas Red (Dead). A) Bright channel; B) Probe channel, λ_{ex}=405 nm, λ_{em}=420-480 nm; C) Live cell channel, λ_{ex}=488 nm, λ_{em}=505-550 nm; D) Dead cell channel, λ_{ex}=570 nm, λ_{em}=585-620 nm; E) Overlay of A, B, C and D.

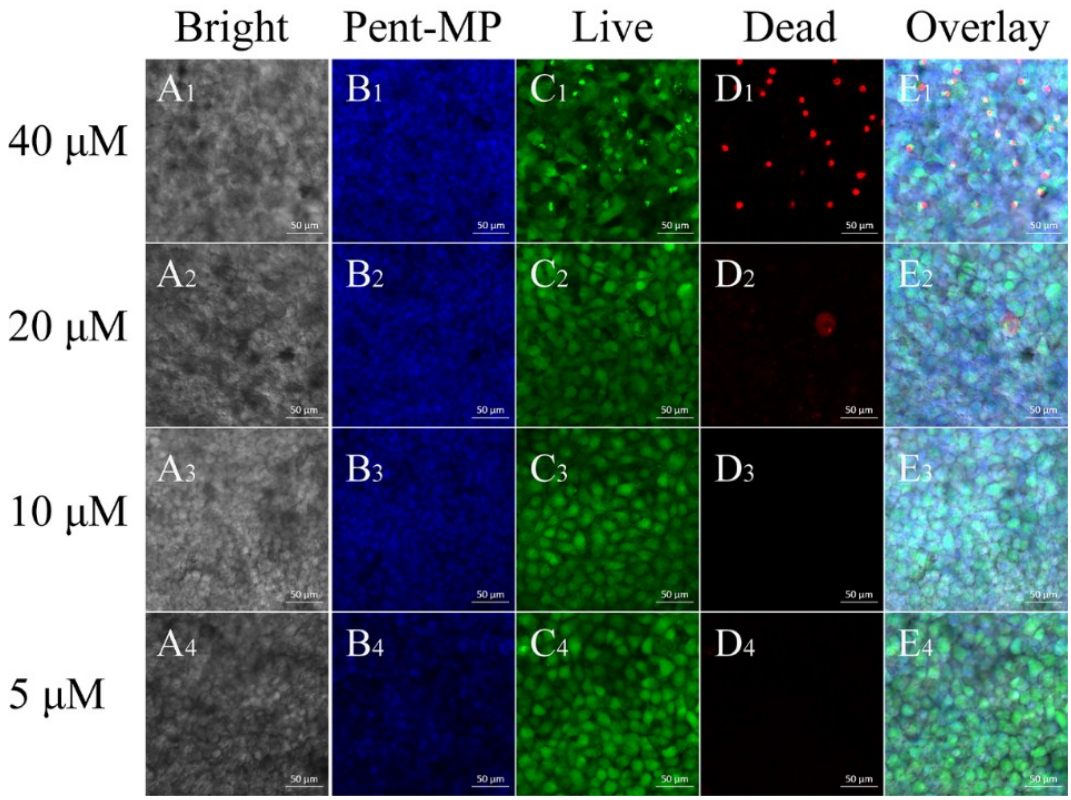


Figure S17 Cell viability of 3D B16 cells treated with different concentration of **Pent-MP** assessed by FITC (Live) and Texas Red (Dead). A) Bright channel; B) Probe channel, λ_{ex}=405 nm, λ_{em}=420-480 nm; C) Live cell channel, λ_{ex}=488 nm, λ_{em}=505-550 nm; D) Dead cell channel, λ_{ex}=570 nm, λ_{em}=585-620 nm; E) Overlay of A, B, C and D.

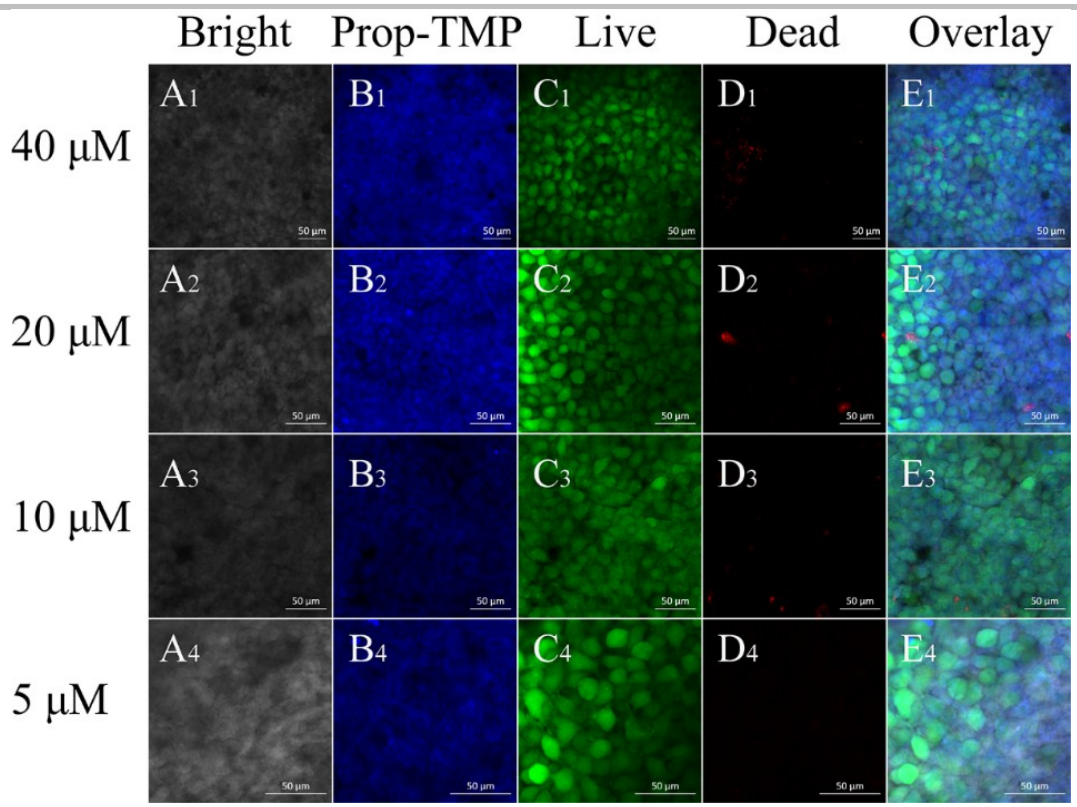
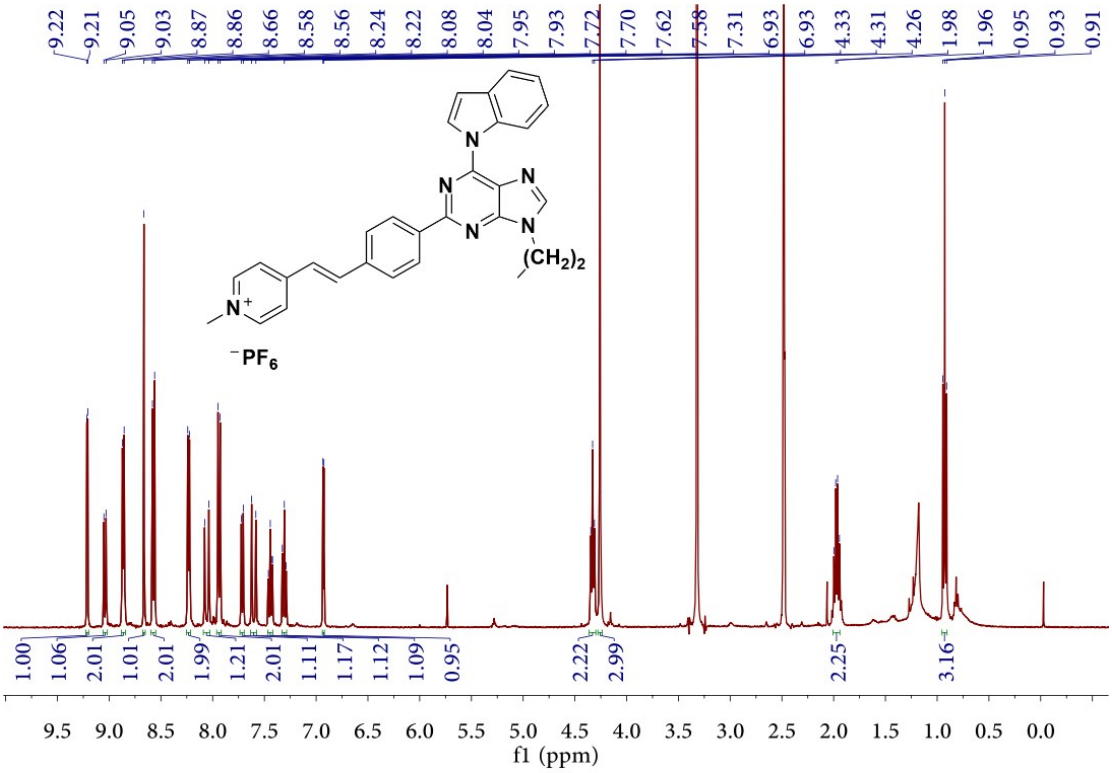


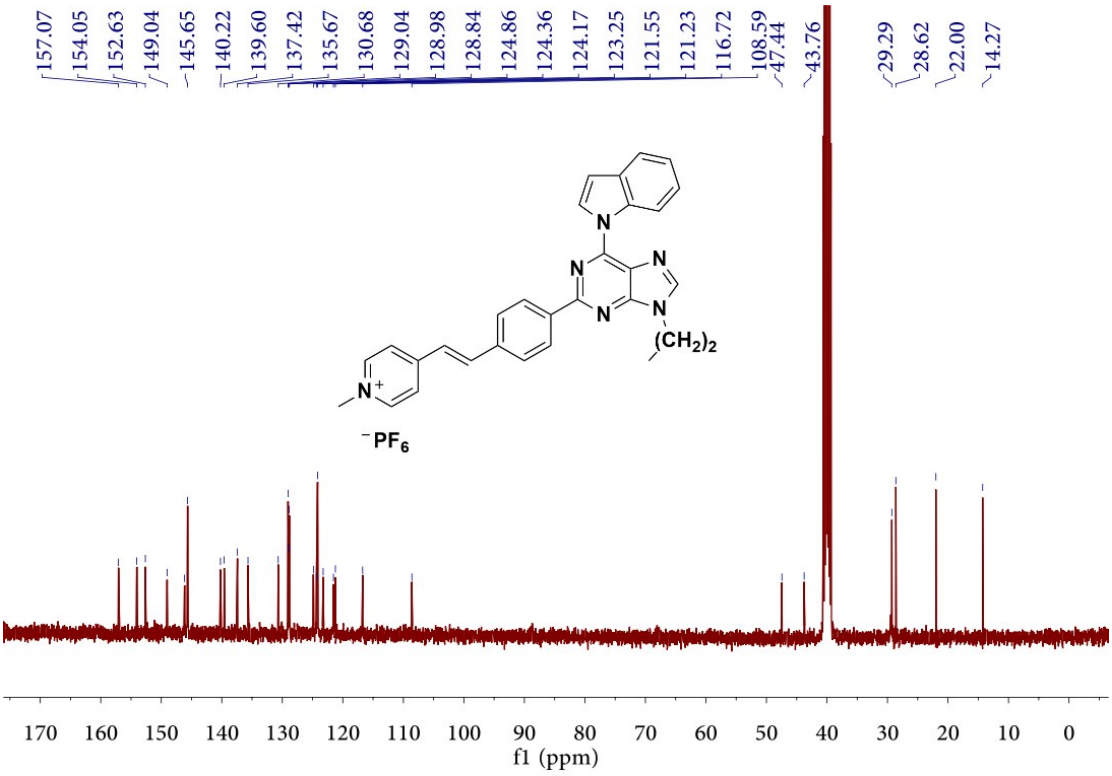
Figure S18 Cell viability of 3D B16 cells treated with different concentration of **Prop-TMP** assessed by FITC (Live) and Texas Red (Dead). A) Bright channel; B) Probe channel, λ_{ex}=405 nm, λ_{em}=420-480 nm; C) Live cell channel, λ_{ex}=488 nm, λ_{em}=505-550 nm; D) Dead cell channel, λ_{ex}=570 nm, λ_{em}=585-620 nm; E) Overlay of A, B, C and D.

To further explore the low cytotoxicity these three compounds really have, the two-colour fluorescence cell viability assay was performed as shown in Figure S15-18. The blue channel represented the light of different probes, the green channel represented the light of fluorescein (FITC) which was a cell-permeable dye for staining of live cells, and the red channel represented the light of Texas Red which was a cell-impermeable dye for staining of dead and dying cells. After 24 hours incubating, while very weak red light could be seen at the concentration of 40 μM, nearly no dead cell could be found at other concentration, which was very match the result of previous cell viability study. These results demonstrated that the good biocompatibility these probes have no matter use in 3D cells imaging.

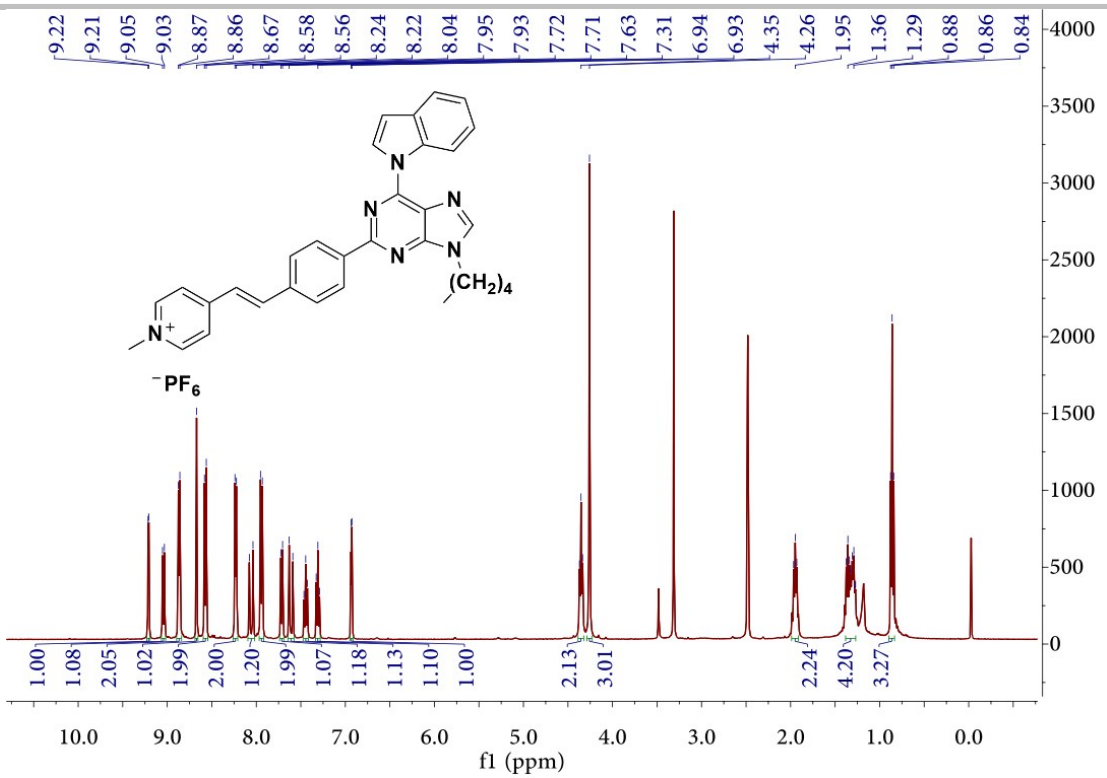
12. NMR Data



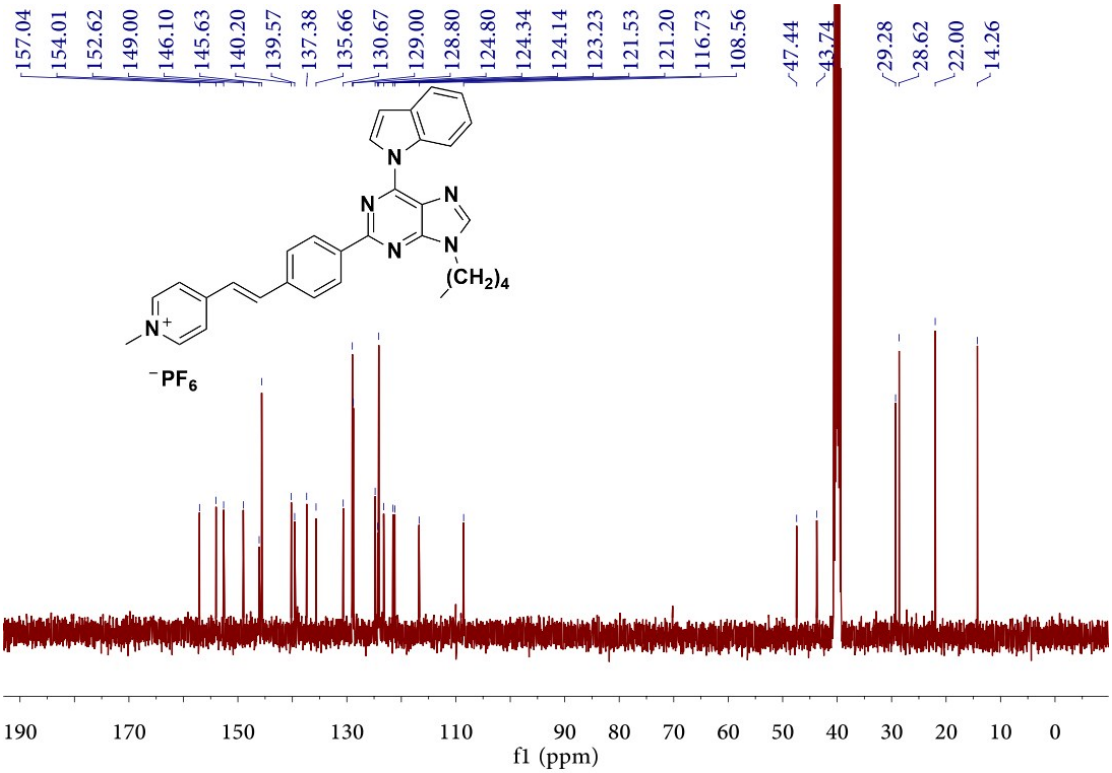
^{1}H NMR of Compound **Prop-MP** in d^{6}-DMSO



^{13}C NMR of Compound **Prop-MP** in d^{6}-DMSO



^{1}H NMR of Compound **Pent-MP** in d^{6}-DMSO



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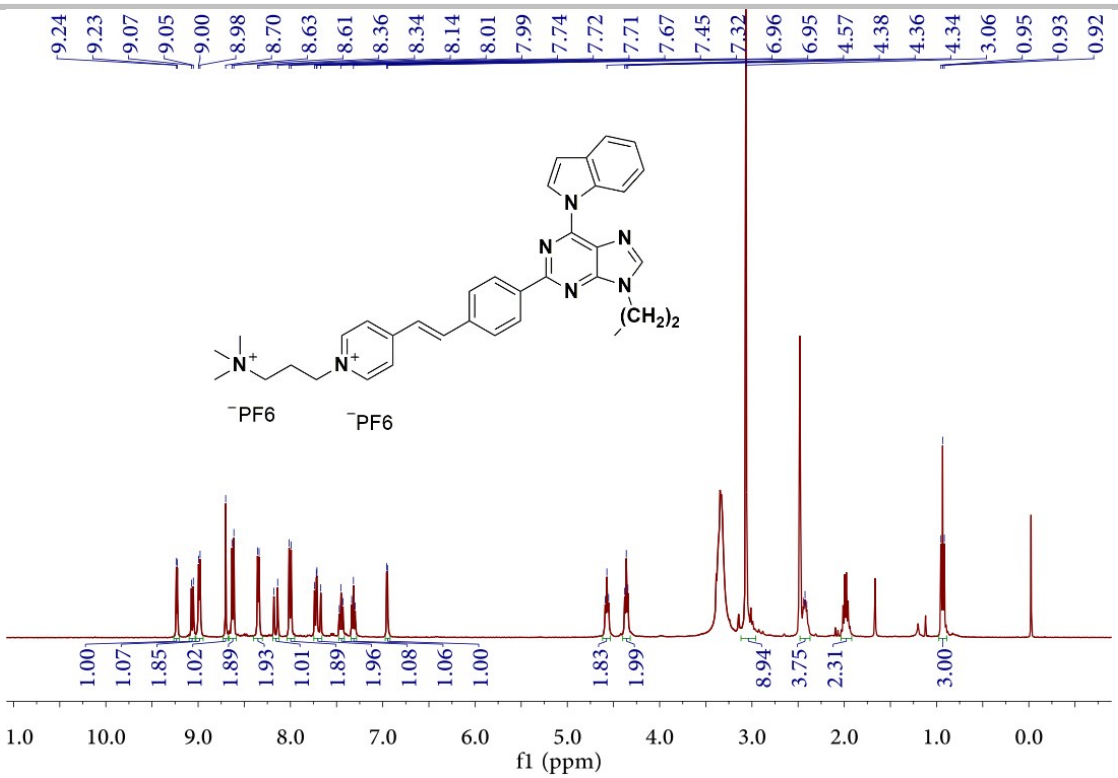
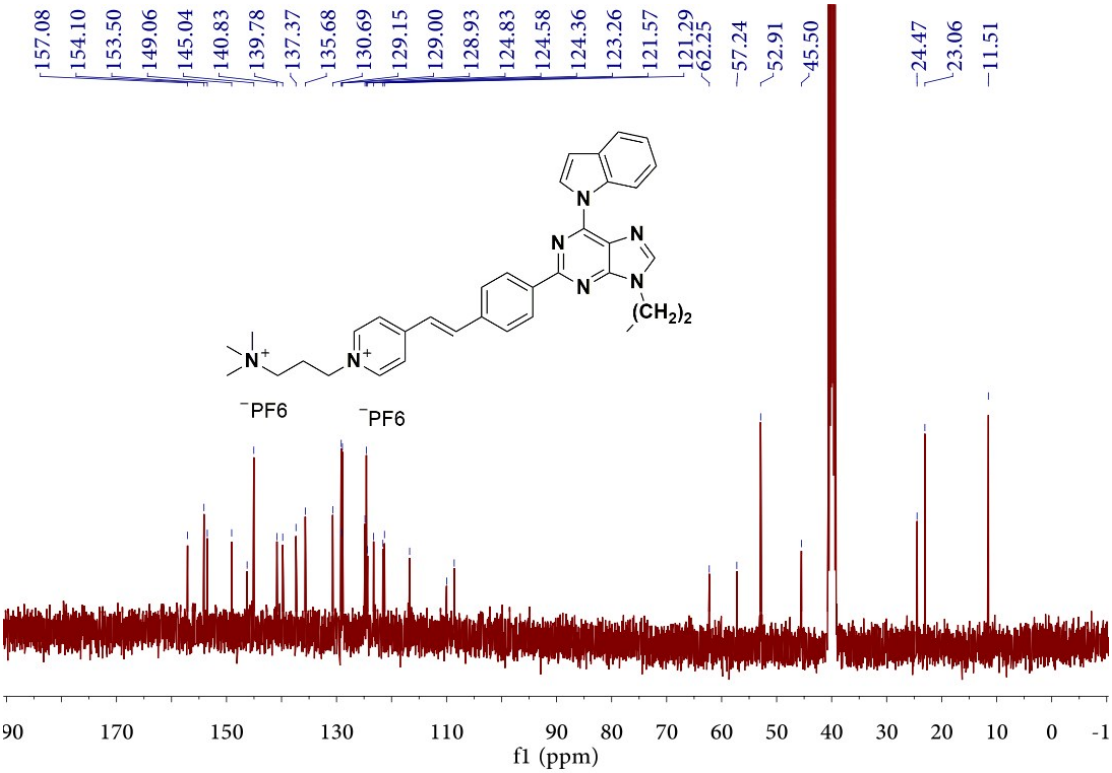


^{1}H NMR of Compound **Prop-TMP** in d^{6}-DMSO



^{1}H NMR of Compound **Prop-TMP** in d^{6}-DMSO

13. ESI-MS Data

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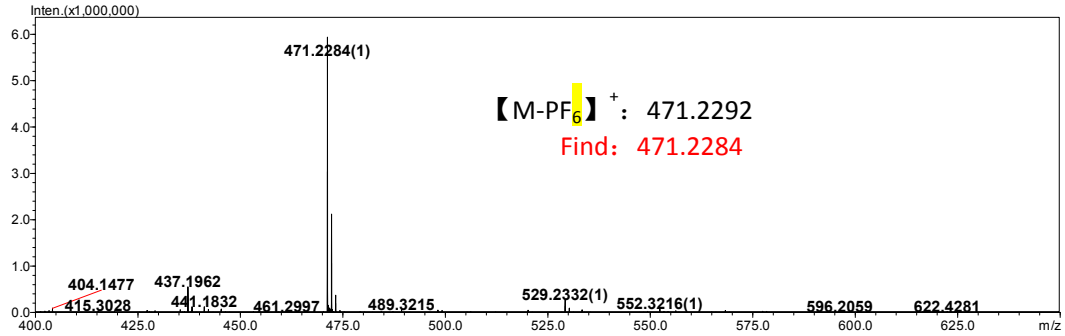

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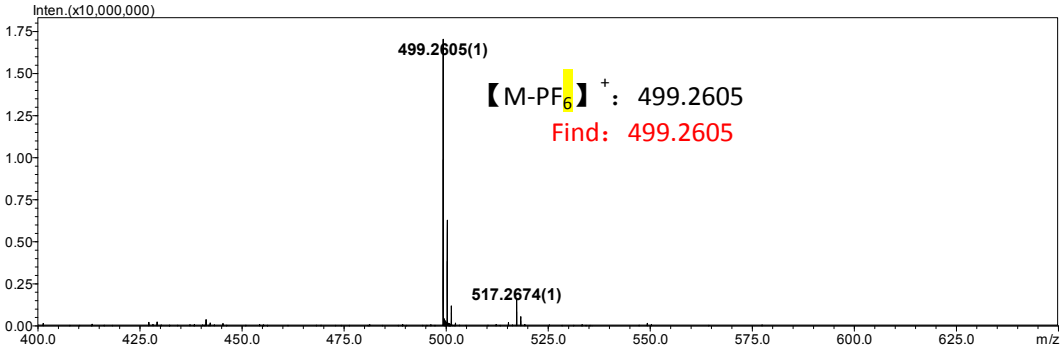

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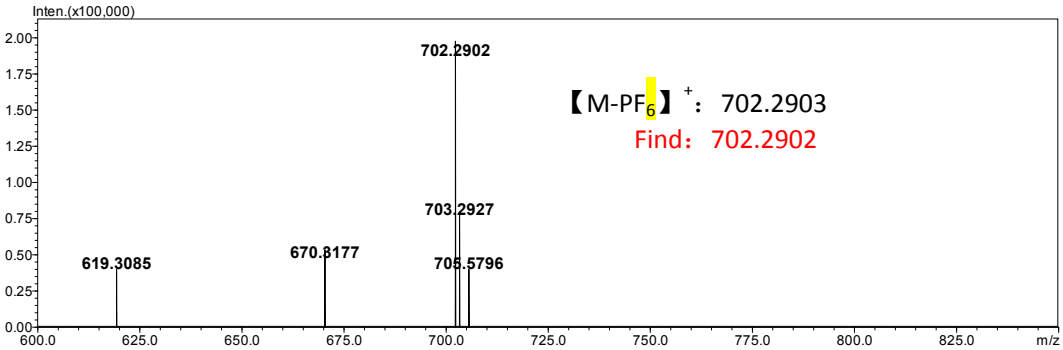

14. References

1. Shi L., Li K., Li L. L., Chen S. C., Li M. Y., Zhou Q., Wang N. and Yu X. Q. *Chem. Sci.*, **2018**, 9, 8969.
2. Wang D., Su H., Kwok R. T. K., Hu X., Zou H., Luo Q., Lee M. M. S., Xu W., Lam J. W. Y. and Tang B. Z. *Chem. Sci.*, **2018**, 9, 3685.
3. C. W. Leung, Y. Hong, J. Hanske, E. Zhao, S. Chen, E. V. Pletneva and B. Z. Tang, *Anal. Chem.*, **2014**,86, 1263
4. Xiao Y. P., Zhang J., Liu Y. H., Huang Z., Wang B., Zhang Y. M. and Yu X. Q. *J. Mater. Chem. B*, **2017**, 5, 8542.