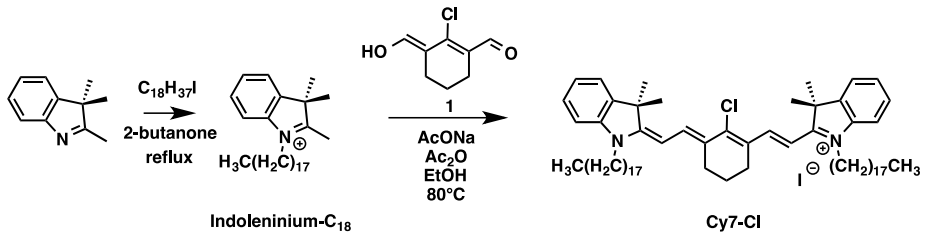
Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

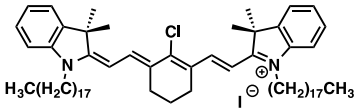
Near Infrared Fluorogenic Probe as a Prodrug Model for Evaluating Cargo Release by Nanoemulsions

Supplementary information

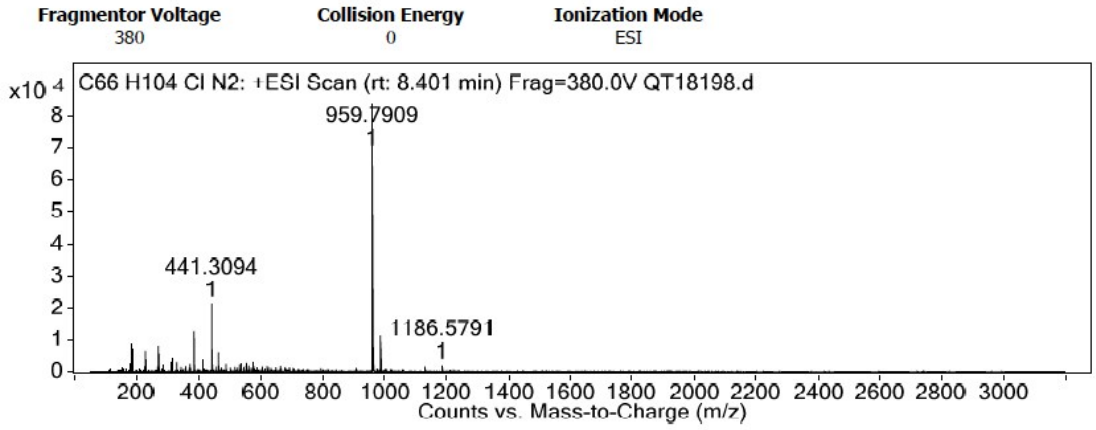
- Synthesis of Cy7-Cl



Indoleninium-C_{18} was synthesized according to a described protocol.[1]

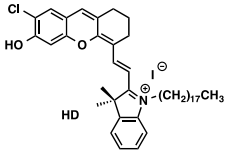


Cy7-Cl. To a solution of 2-chloro-3-(hydroxymethylene) cyclohexene-1-carbaldehyde **1** (164 mg, 0.950 mmol) and Indolenine-C_{18} (1.023 g, 1.900 mmol, 2 eq) in EtOH (5 mL) was added sodium acetate (78 mg, 0.95 mmol, 1 eq) and acetic anhydride (1 mL). The mixture was heated at 80°C for 30 min. The reaction was monitored by TLC: DCM/MeOH (95/5). After cooling down, EtOH (5 mL) was added and the product was allowed to crystallize overnight. The solution was filtered and washed with EtOH and Et_2O and dried under vacuum to obtain 370 mg (Yield=36%) of **Cy7-Cl** as a shiny green powder. **Cy7-Cl** was found to be insoluble in various deuterated solvents and thus could not be characterized by NMR spectroscopy. Therefore it was involved in the next step without further characterizations. HRMS (ESI^+), calcd for $C_{66}H_{104}ClN_2^+$ $[M]^+$: 959,7883, found 959.7909.

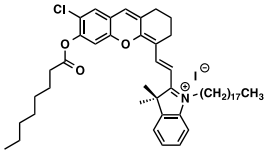


HRMS spectrum of Cy7-Cl

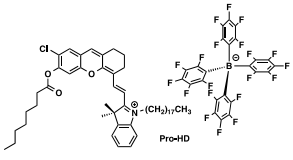
- Characterizations of products



HD. To a solution of **Cy7-Cl** (368.2 mg, 0.338 mmol) and 4-chlororesorcin (195.4 mg, 4 eq, 1.352 mmol) in dry DMF (3 mL) was added triethylamine (0.5 mL). The solution was allowed to stir under argon at 75°C for 2 h. The solvents were evaporated. The product was extracted with DCM and washed with water and brine. The organic phase was dried over anhydrous $MgSO_4$, filtered and evaporated. The crude was purified by column chromatography on silica gel using DCM/MeOH, 95:5 to obtain 111 mg of HD (Yield= 42%) as a blue powder. Rf = 0.25, DCM/MeOH, 95:5. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.11 (d, *J* = 13.4 Hz, 1H, H Ar), 7.43 (s, 1H, H Ar), 7.31-7.29 (m, 2H, H Ar), 7.08 (td, *J* = 7.4, 0.6 Hz, 1H, H Ar), 6.84 (d, *J* = 7.8 Hz, 1H, H Ar), 6.71 (s, 1H, HC=C), 5.65 (d, *J* = 13.5 Hz, 1H, HC=C), 3.79 (t, *J* = 7.5 Hz, 2H, H_2C-N), 2.72-2.69 (m, 2H, CH_2), 2.64-2.61 (m, 2H, CH_2), 1.96-1.90 (m, 2H, CH_2), 1.77 (t, *J* = 6.8 Hz, 2H), 1.69 (s, 6H, 2 CH_3), 1.44-1.27 (m, 32H, 19 CH_2), 0.90 (m, *J* = 6.9 Hz, 3H, CH_3). ^{13}C-NMR (126 MHz, $CDCl_3$): δ 160.19 (CN^+), 157.83 (C-O), 143.30, 139.74, 139.45, 133.14, 132.16, 128.11, 127.19, 122.39, 122.14, 116.47, 115.68, 115.36, 108.06, 103.80, 94.37, 94.34, 47.59, 43.20, 31.93, 29.70, 29.36, 28.65, 27.96, 27.10, 26.51, 24.41, 22.70, 21.30, 14.13. HRMS (ESI^+), calcd for $C_{43}H_{59}NO_2Cl$ $[M]^+$: 656.4229, found 656.4224.



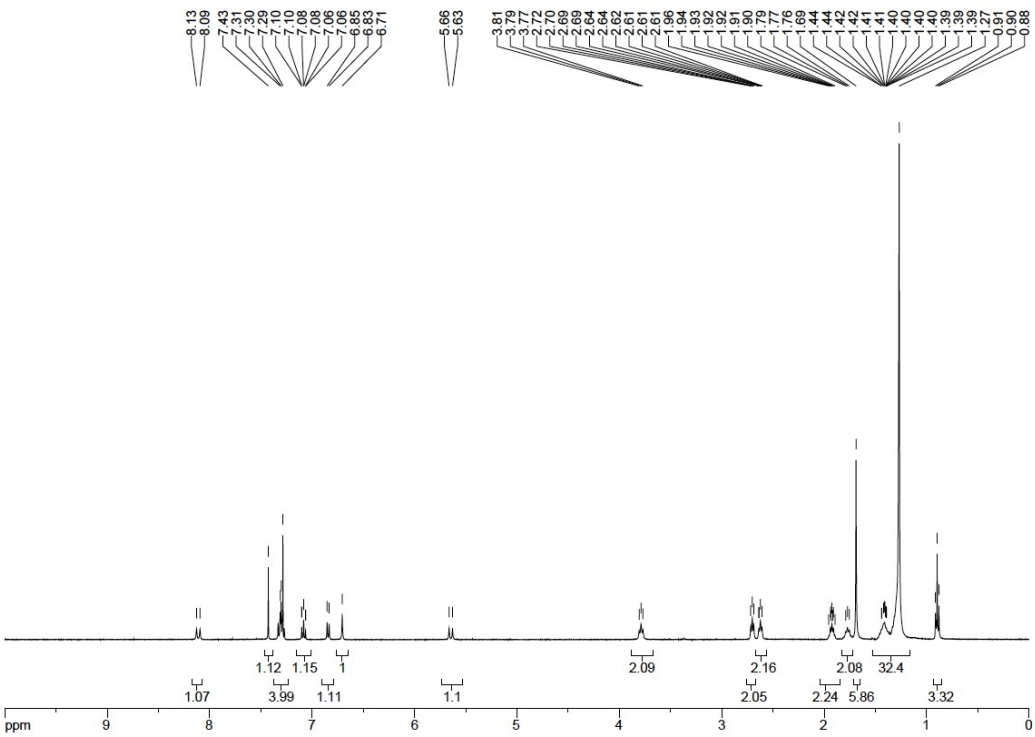
pro-HD-I$^-$. To a solution of caprylic acid (37.4 g, 3 eq, 0.230 mmol), EDC (47.5 mg, 4 eq, 0.306 mmol) and DMAP (3.66 mg, 0.13 eq, 0.03 mmol) in DCM (3 mL) was added **HD** (60 mg, 1 eq, 0.076 mmol) in DCM (2mL). The reaction was allowed to stir overnight at room temperature. The solvents were evaporated and the crude was purified by column chromatography on silica gel using DCM/MeOH, (95:5) to obtain 28 mg of **pro-HD** (Yield= 14%) as a blue syrup. Rf = 0.1, DCM/MeOH, (95:5). ^{1}H-NMR (400 MHz, $CDCl_3$, MeOD): δ 8.73 (d, *J* = 15.2 Hz, 1H, HC=C), 7.71-7.51 (m, 5H, H Ar), 7.38 (s, 1H, H Ar), 7.20 (s, 1H, H Ar), 6.68 (d, *J* = 15.3 Hz, 1H, HC=C), 4.46 (t, *J* = 7.3 Hz, 2H, CH_2N^+), 2.79-2.67 (m, 6H, 2 CH_2), 1.95-1.93 (m, 4H, 2 CH_2), 1.82-1.77 (m, 8H, 1 CH_2, 2 CH_3), 1.47-1.23 (m, 38H, 19 CH_2), 0.93-0.87 (m, 6H, 2 CH_3). ^{13}C-NMR (126 MHz, $CDCl_3$): δ 179.0 (CN^+), 171.0 (C Ar), 158.2, 151.30, 148.35, 146.12, 142.43, 141.38, 131.69, 129.40, 128.26, 128.21, 127.61, 123.44, 122.49, 120.79, 116.29, 113.82, 111.43, 108.51, 51.14, 47.08, 34.06, 31.91, 31.64, 29.69, 29.67, 29.65, 29.61, 29.57, 29.41, 29.38, 29.34, 29.04, 28.87, 28.45, 28.07, 26.88, 24.76, 24.61, 22.67, 22.58, 20.19, 14.10, 14.06. HRMS (ESI^+), calcd for $C_{51}H_{73}NO_3Cl$ $[M]^+$: 782.5273, found 782.5294.



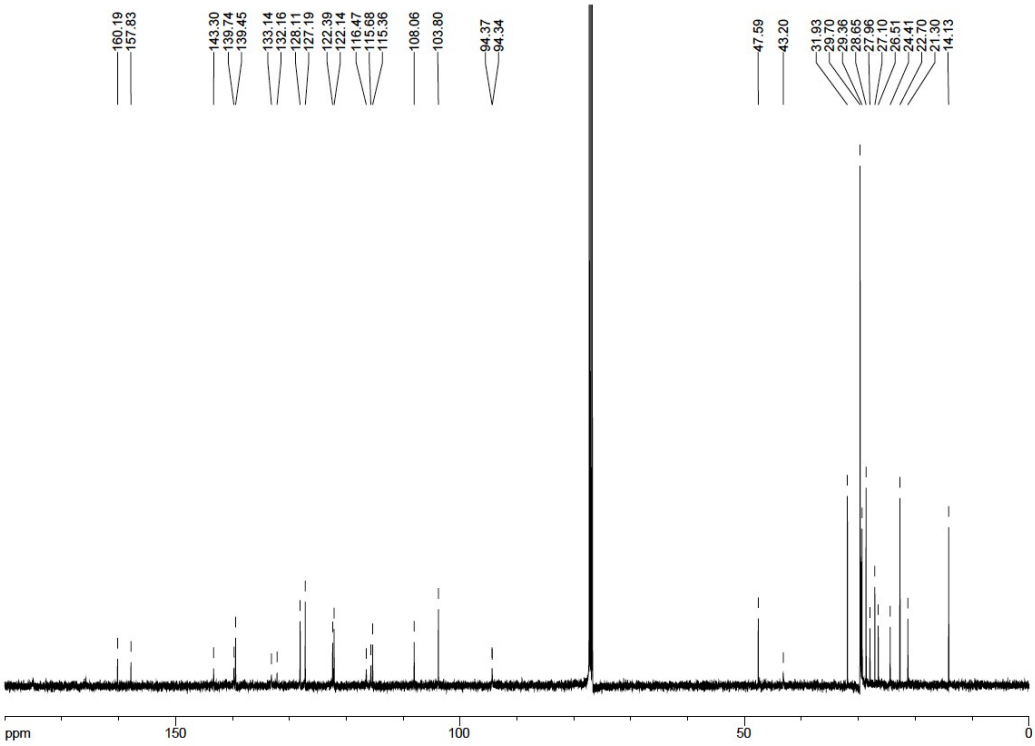
Pro-HD. To a solution of pro-HD-I$^-$ (20 mg, 0.025 mmol) in DCM (2 mL) was added Lithium tetrakis(pentafluorophenyl)borate lithium salt (TPB-F_5-Li) (51 mg, 0.075 mmol, 3 eq). The solution was allowed to stir at room temperature for 5 min and, after a control TLC, the product was purified by column chromatography on silica gel (DCM/MeOH, 95:5) to give 33 mg of Pro-HD with a quantitative yield. Pro-HD displayed similar ^{1}H and ^{13}C NMR and HRMS spectra than Pro-HD-I$^-$.^{19}F-NMR (376 MHz, $CDCl_3$): δ -132.52 (d, *J* = 10.5 Hz, 1F), -163.07 (t, *J* = 20.6

Hz, 1F), -166.81 (t, *J* = 17.8 Hz, 1F). ^{11}B-NMR (128 MHz, $CDCl_3$): δ -16.69 (s, 1B). HRMS (ESI^+), calcd for $C_{51}H_{73}NO_3Cl$ $[M]^+$: 782.5273, found 782.5287.
^{1}H, ^{13}C, ^{19}F and ^{11}B NMR spectra as well as HRMS can be found below.

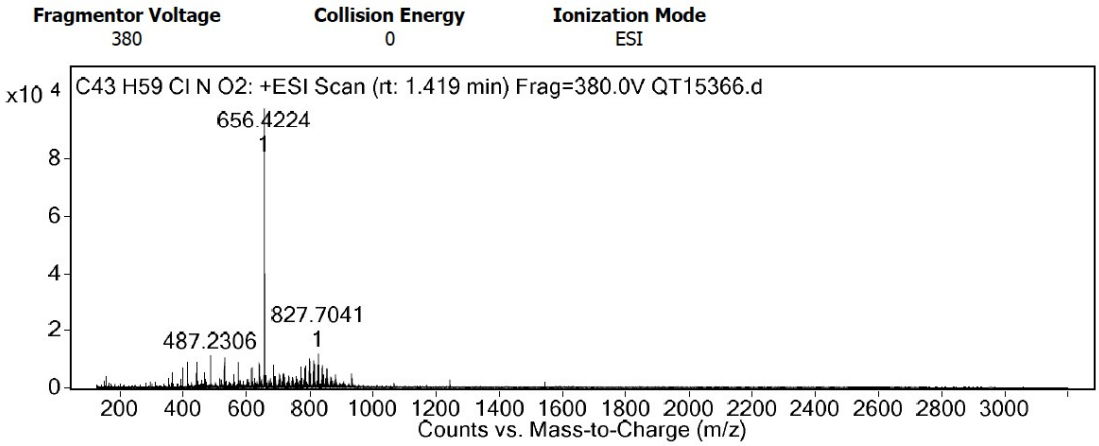
- NMR and mass spectra



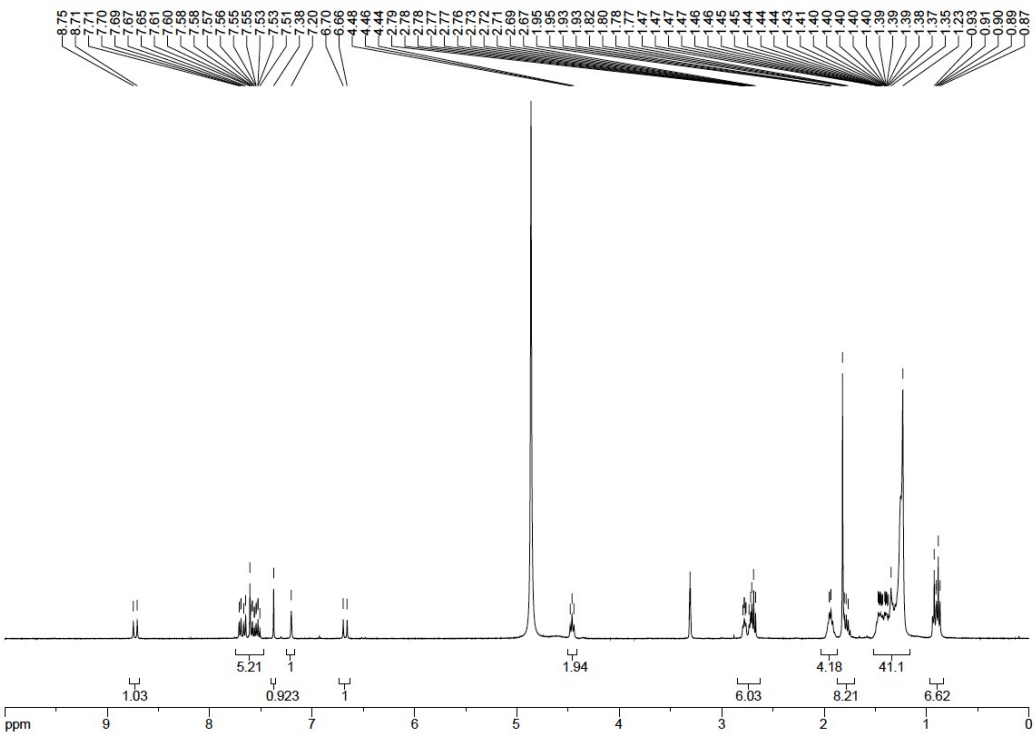
^{1}H NMR spectrum of HD ($CDCl_3$)



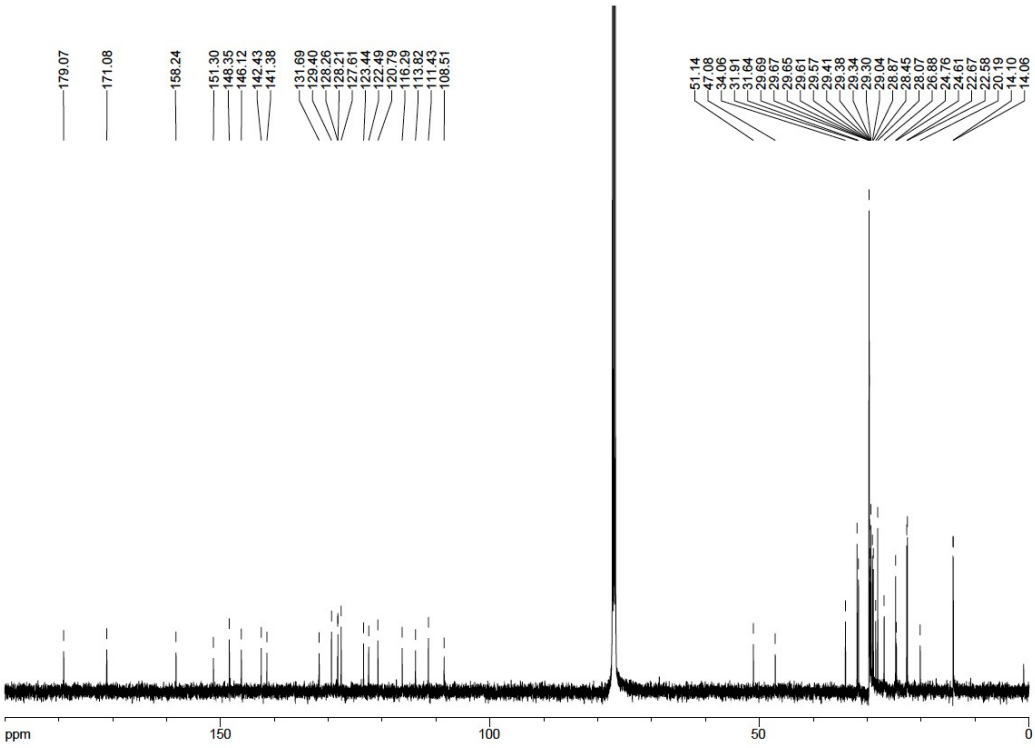
^{13}C NMR spectrum of HD ($CDCl_3$)



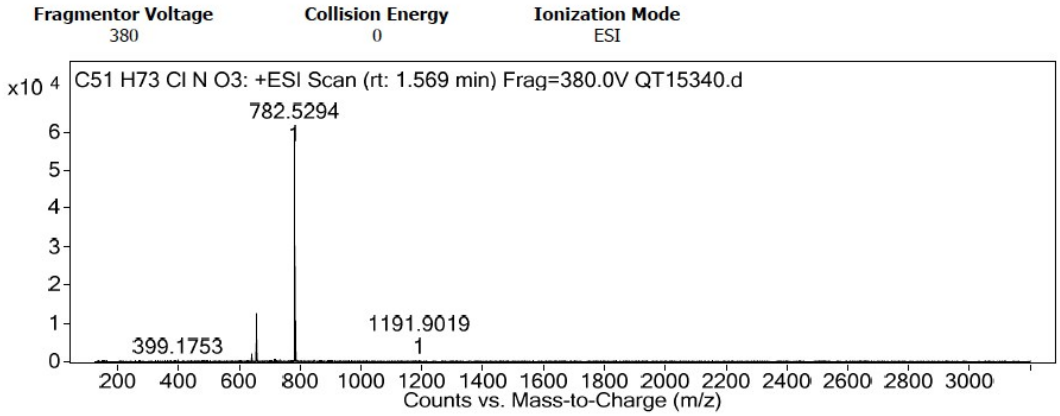
HRMS spectrum of HD (by infusion)



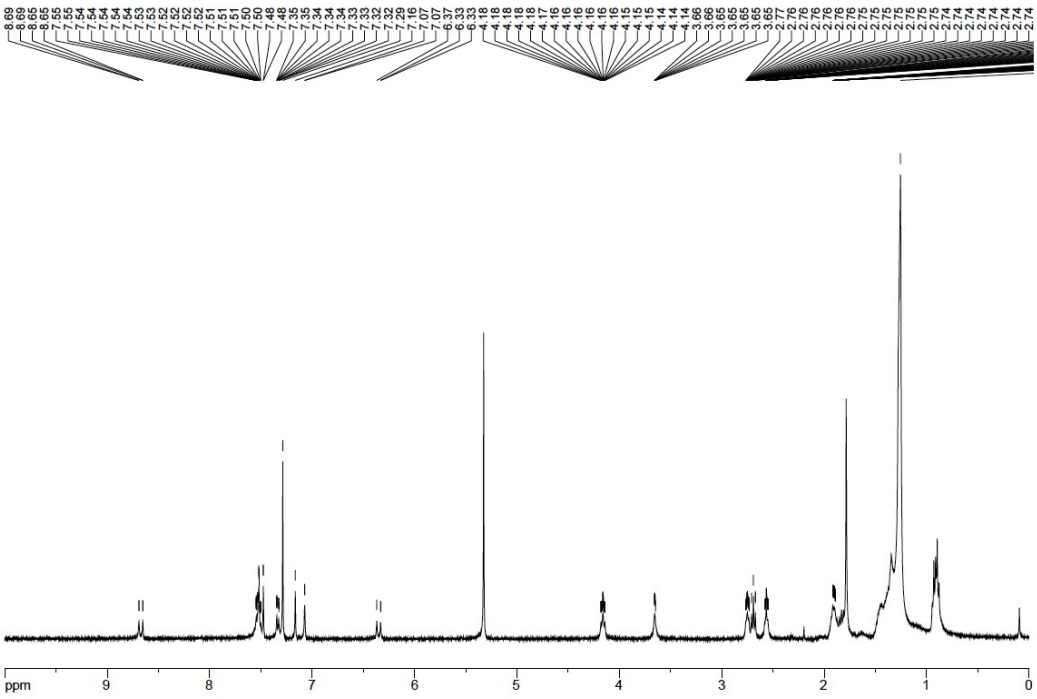
^{1}H NMR spectrum of Pro-HD-I^{-} ($CDCl_3$)



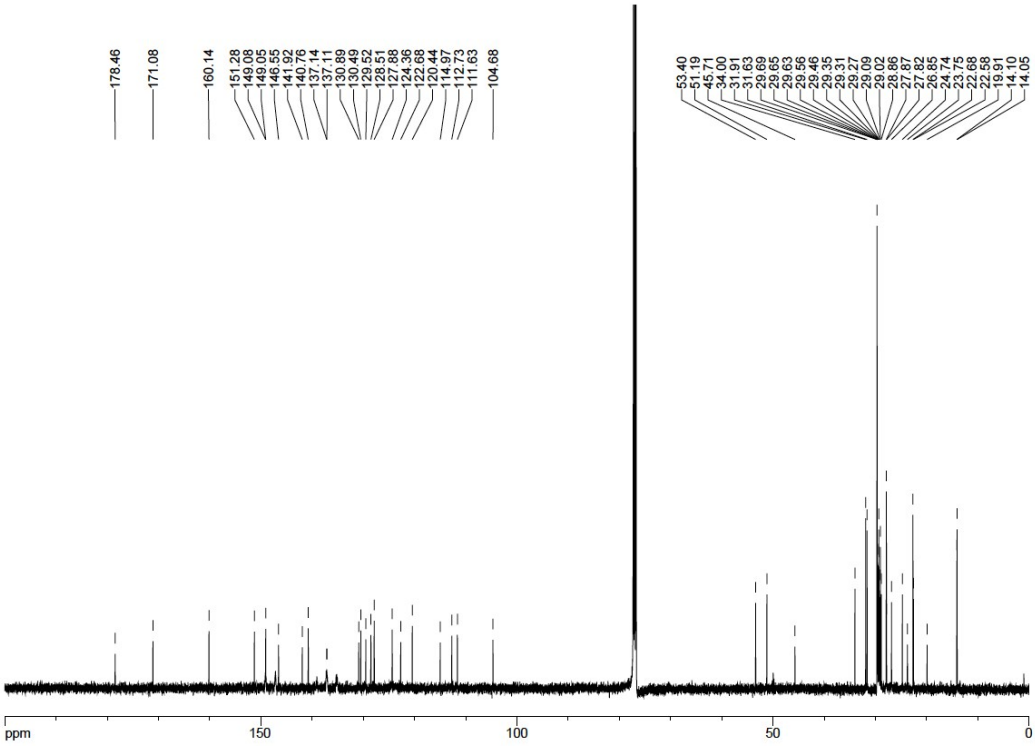
^{13}C NMR spectrum of Pro-HD-I^{-} ($CDCl_3$)



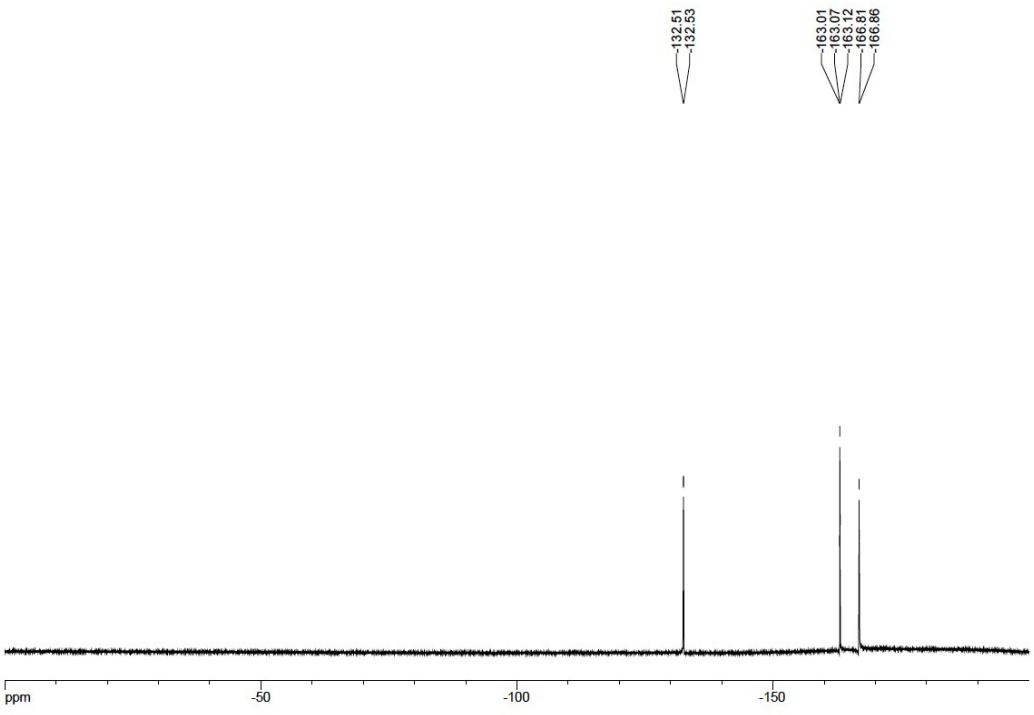
HRMS spectrum of Pro-HD-I^- (by infusion)



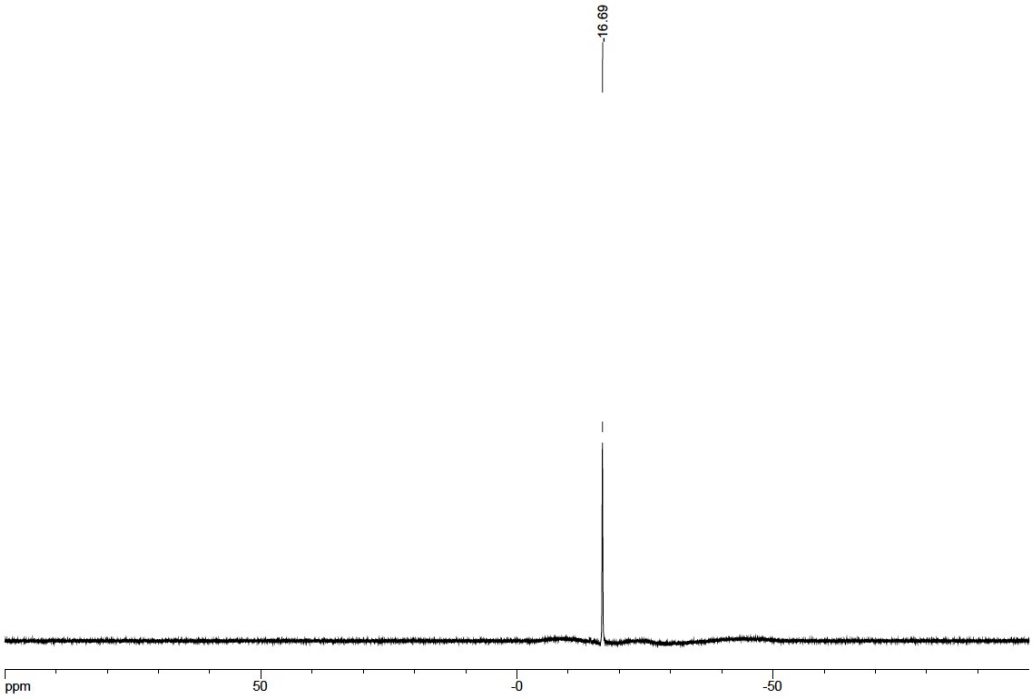
^{1}H NMR spectrum of Pro-HD ($CDCl_3$)



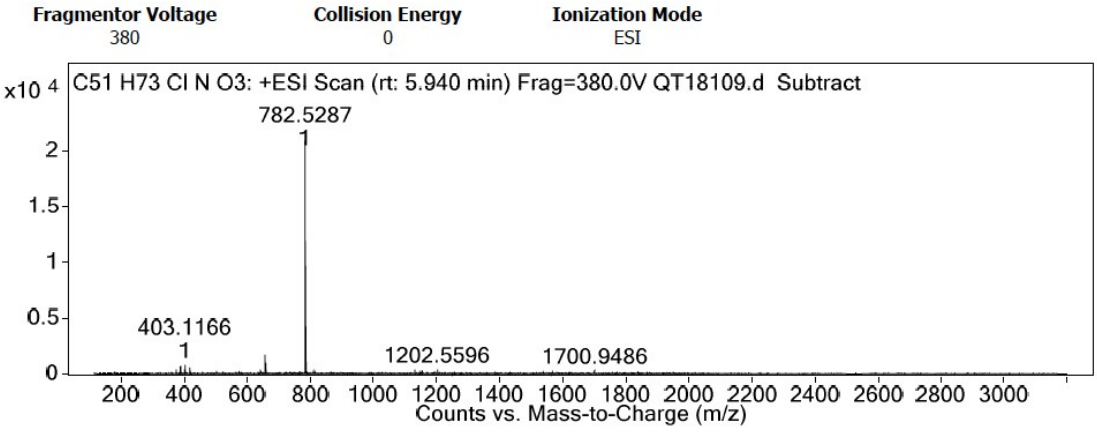
^{13}C NMR spectrum of Pro-HD ($CDCl_3$)



^{19}F NMR spectrum of Pro-HD ($CDCl_3$)



^{11}B NMR spectrum of Pro-HD ($CDCl_3$)



HRMS spectrum of Pro-HD-$TPBF_5$ (by infusion)

Spectroscopy

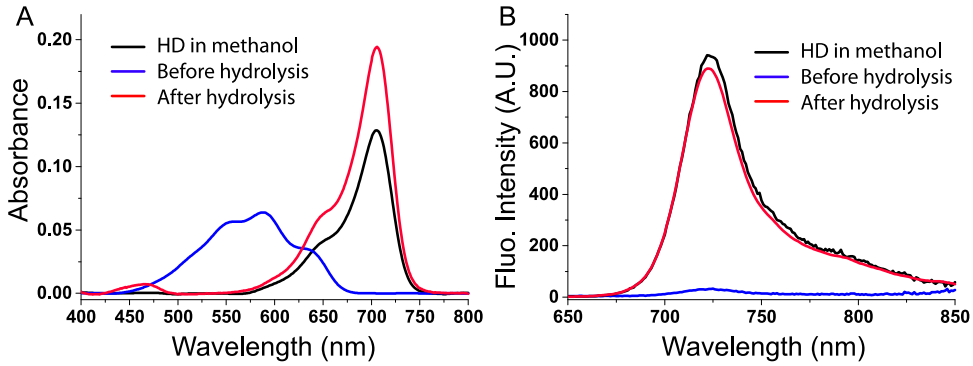


Figure S1. Absorption (A) and emission spectra (B) of Pro-HD before and after hydrolysis compared to HD in pure methanol. After hydrolysis of Pro-HD, HD displayed and enhanced absorption due to the presence of NaOH that favors the phenolate form of HD.

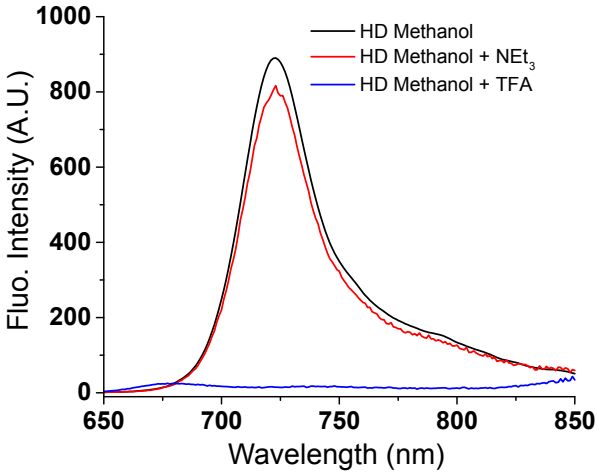


Figure S2. Fluorescence spectra of HD in methanol and in the presence of a base (triethylamine, 5 µL in 1 mL MeOH, 69 ?M) and an acid (TFA, 5 µL in 1 mL MeOH: 29 mM).

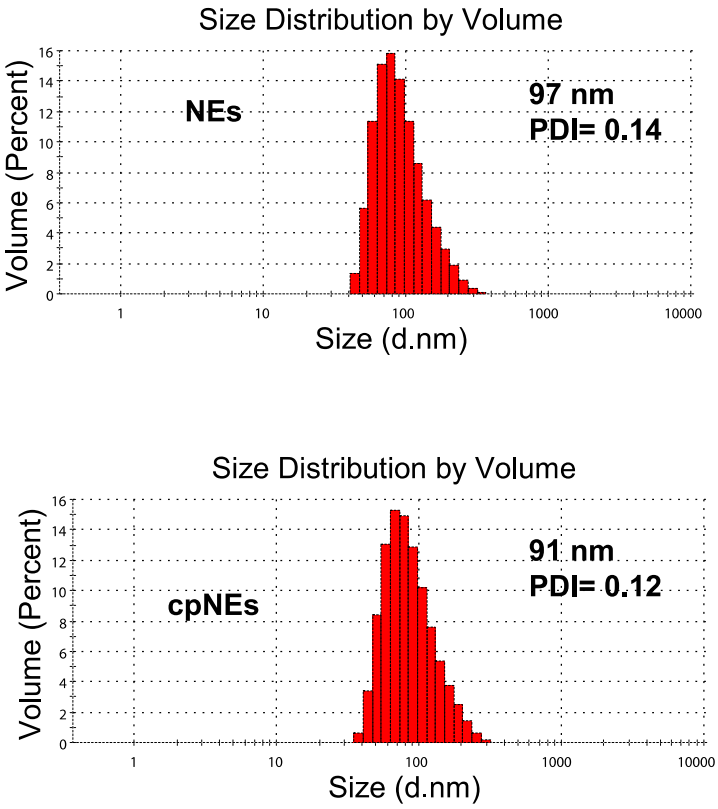


Figure S3. Histogram of size distribution of Pro-HD loaded NEs and cpNEs obtained by DLS measurements.

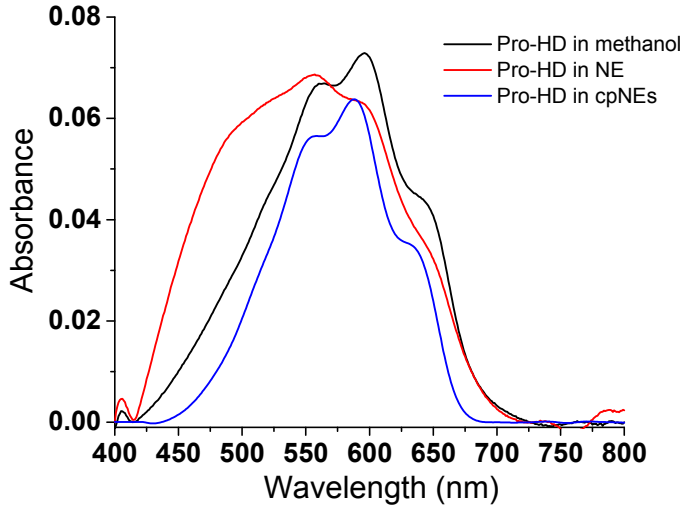


Figure S4. Absorption spectra of Pro-HD (1 μM) in methanol and in NEs and cpNEs loaded at 0.5 wt %.

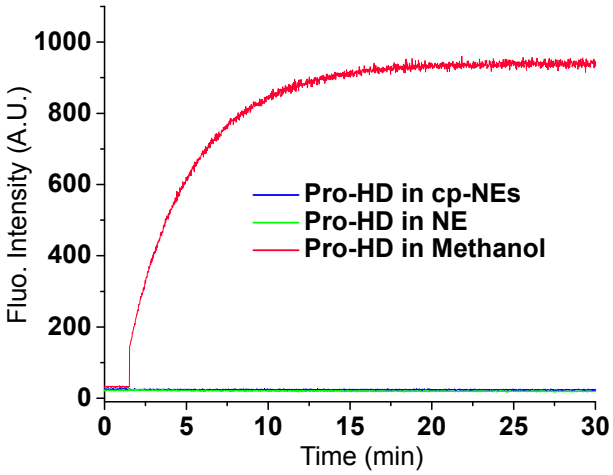


Figure S5. Evolution of the fluorescence intensity at 723 nm over 30 min and after addition of aqueous NaOH (at 90 s, final concentration was 250 μM). Whereas Pro-HD is hydrolyzed in methanol, it is protected in NEs. Excitation wavelength was 640 nm.

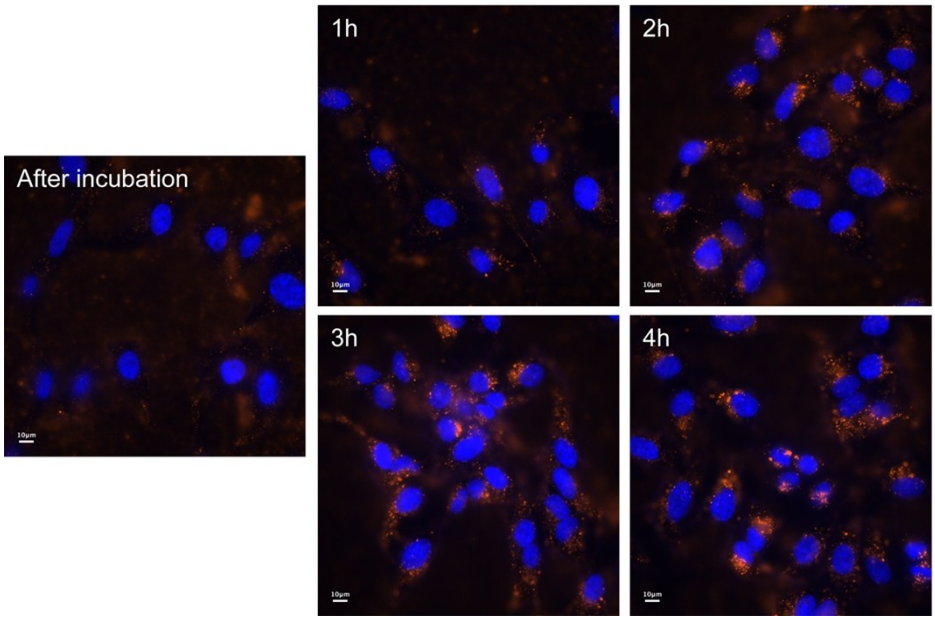


Figure S6. Epi-fluorescence microscopy imaging of fixed HeLa cells after pre-incubated for 2 h in the presence of pro-HD (1 μM) and after washing and 1, 2, 3 and 4h of further incubation. **HD** was excited at 641 nm. The nucleus was stained with Hoechst (5 μg.mL^{-1}). Scale bar is 10 μm.

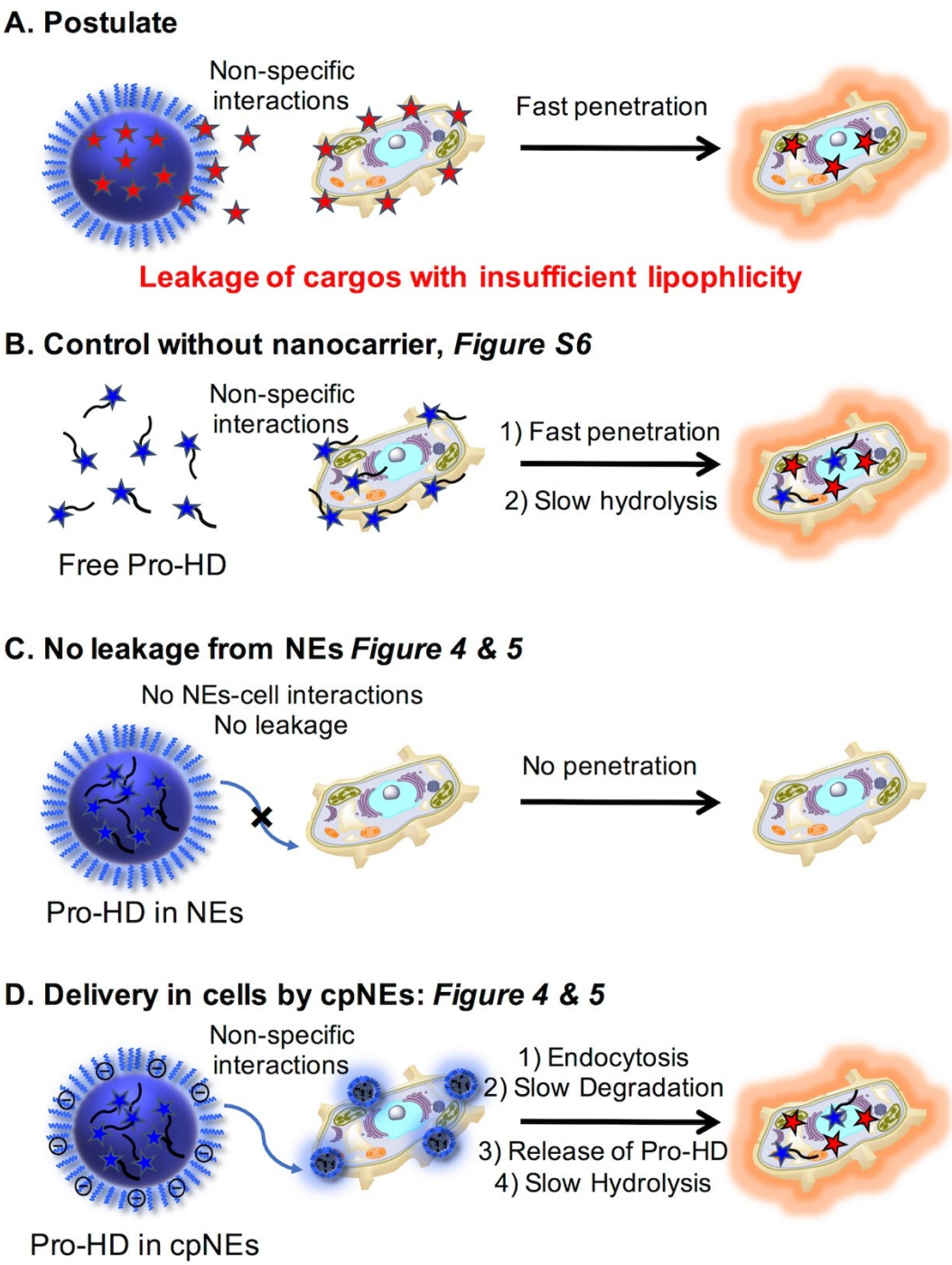


Figure S7. Summary scheme. (A) Initial postulate: cargos with insufficient lipophilic nature leak out from NEs but is sufficiently lipophilic to interact and penetrate the cells.[2, 3, 4, 5] (B) Pro-HD is lipophilic and penetrate quickly in cells by non-specific interactions, within 2 h the maximum fluorescence is obtained proving that Pro-HD can be hydrolysed in cells. (C) Similarly to our previous work,[2] Pro-HD when encapsulated in NEs is stable and does not leak out from NEs. (D) As already demonstrated,[6] similarly to NEs, cpNEs are able to retain lipophilic cargos but can penetrate in cells by non-specific interactions. Consequently, the mechanism of release is not controlled by diffusion (leakage) but by subsequent: endocytosis, degradation of the NEs' matrix, release of Pro-HD and finally hydrolysis into HD.

References

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(2) Kilin, V. N.; Anton, H.; Anton, N.; Steed, E.; Vermot, J.; Vandamme, T. F.; Mely, Y.; Klymchenko, A. S. Counterion-Enhanced Cyanine Dye Loading into Lipid Nano-Droplets for Single-Particle Tracking in Zebrafish. *Biomaterials* **2014**, *35* (18), 4950–4957. https://doi.org/10.1016/j.biomaterials.2014.02.053.
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