Supporting information

Importance of the physicochemical properties of fluorescent dyes for the *in vivo* biodistribution of membrane-permeable macromolecular imaging probes

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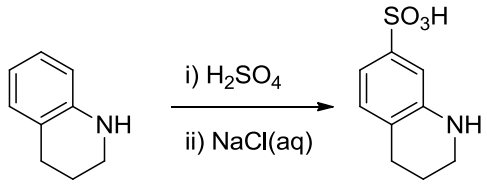
Contents

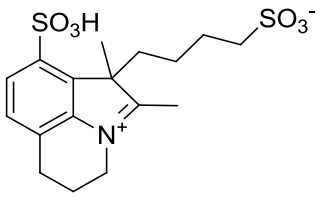
Synthesis of PBI3920 conjugating Halotag ligand

Sulfonation of *o*-toluidine under acid conditions leads to a single isomer product in which the sulfonate group is *para* to the amine [1]. We were expecting such results in the sulfonation reaction of 1,2,3,4-tetrahydroquinoline. However, the product ^{1}H NMR spectrum of the subsequent Fisher indole synthesis was not indicative of the 8-sulfonate. To absolutely define the correct isomer of the sulfonation reaction of 1,2,3,4-tetrahydroquinoline, we performed a x-ray crystal structure determination of this product (Figure S2, Table S1 and S2). The unexpected 7-isomer as shown below (compound 1) was confirmed.

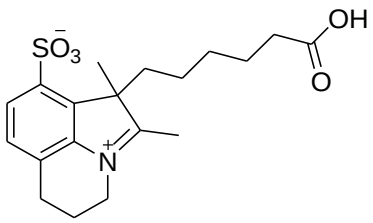
Compound 1



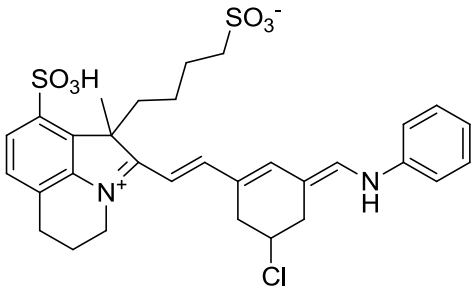
Sodium 1,2,3,4-tetrahydroquinoline-7-sulfonate. 1,2,3,4-tetrahydroquinoline was added dropwise to 40 mL fuming sulfuric acid at 0°C over 15 minutes. This mixture was then heated to 100°C for 1 hour with stirring. After cooling to 0°C saturated NaCl (aqueous) was added drop wise, precipitating the crude product as a white solid. The solid was washed, first with cold isopropanol, followed by diethyl ether, and then dried under vacuum to give the desired product as a white solid. ^{1}H-NMR (300 MHz, d_6 DMSO): δ 7.43 (m, 2H), 7.21 (d, 1H), 3.38 (m, 2H), 2.81 (t, 2H) 1.96 (m, 2H) ppm; MS *m/z* calculated for $C_9H_{10}NO_3S$ (M-): 212.04. Found 212.0 (M-, ESI-).



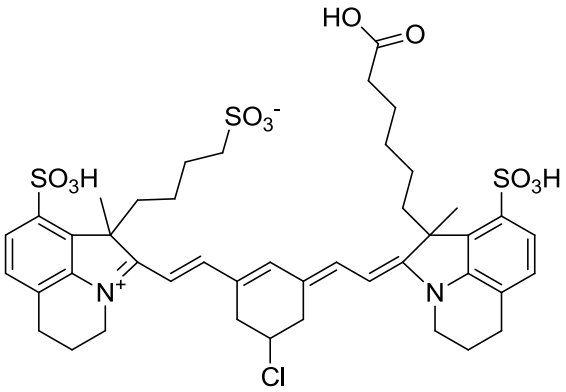
4-(5,6-Dimethyl-1,2,3,6-tetrahydropyrrolo[3,2,1-*ij*]quinolinium-6-yl)butane-1-sulfonate. 3,4-Dihydroquinolin-1(2H)-amine (2.0 g, 13.5 mmol) and 5-methyl-6-oxoheptane-1-sulfonic acid [2] (4.216 g, 20.2 mmol) were refluxed in 15 mL acetic acid and 1.5 mL concentrated HCl for 1 hr. The mixture was cooled and volatiles were removed under reduced pressure giving a brown residue that was purified by flash chromatography to give the product (3.4 g, 78.4%) as a yellow solid. ^{1}H-NMR (300 MHz, D_2O) δ: 7.53 (m, 2H), 7.42 (m, 1H), 4.44 (m, 2H), 3.02 (t, *J* = 6 Hz, 2H), 2.74 (m, 5H), 2.33-2.21 (m, 4H), 1.59 (m, 5H), 0.93 (m, 2H).



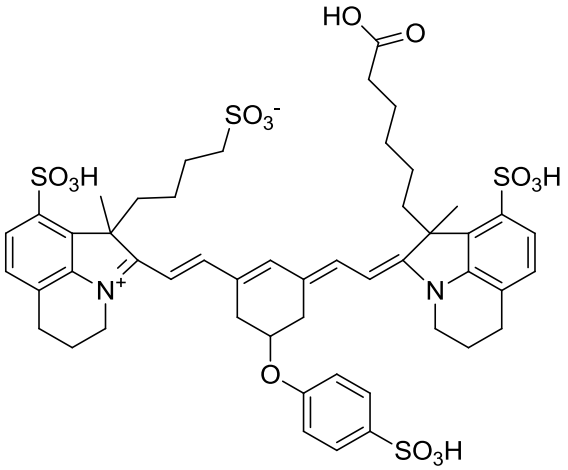
6-(5-Carboxypentyl)-5,6-dimethyl-1,2,3,6-tetrahydropyrrolo[3,2,1-*ij*]quinolinium-7-sulfonate. 1,2,3,4-tetrahydroquinoline-7-sulfonate (1.72 g, 7.30 mmol) was suspended in 8 mL glacial acetic acid with stirring. To this was added $NaNO_2$ (611.0 mg, 8.86 mmol) in 10 mL water dropwise over 15 minutes. The reaction was stirred for 45 minutes at room temperature. 7-methyl-8-oxononanoic acid (4.8 mL, 45 mmol) was subsequently added followed by Zn dust (1.58 g, 24.2 mmol) which was added in small portions. The reaction mixture was heated to reflux for 1 hour, cooled to room temperature, and volatiles were removed under reduced pressure. Isopropanol was added to the residue which was stirred overnight precipitating the product. A grey solid (500 mg, 18.4%) that was collected by vacuum filtration. ^{1}H-NMR (300 MHz, CD_3OD): δ 8.00(d, 1H), 7.50 (d, 1H), 4.50 (m, 2H), 3.35 (m, 3H), 3.10 (m, 2H), 3.05 (t, 2H), 2.25 (m, 2H), 1.81 (s, 3H), 1.40 (m, 2H), 1.15 (m, 2H), 0.60 (m, 2H) ppm; MS *m/z* calculated for $C_{19}H_{26}NO_5S$ (M+2H): 382.2. Found 383.2 (M+2H, ESI+).



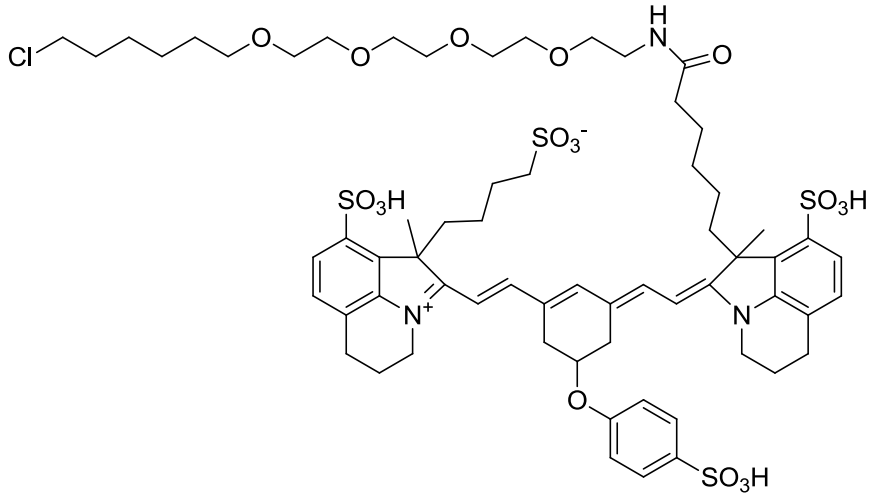
2-((*E*)-2-((*E*)-5-chloro-3-((phenylamino)methylene)cyclohex-1-en-1-yl)vinyl)-1-methyl-9-sulfo-1,4,5,6-tetrahydropyrrolo[3,2,1-*ij*]quinolin-3-ium-1-yl)butane-1-sulfonate. Indolene (1.0 g, 2.49 mmol) and *N*-[(3-(Anilinomethylene)-2-chloro-1-cyclohexen-1-yl)methylene]aniline monohydrochloride (0.89 g, 2.49 mmol) were suspended in absolute ethanol at 60°C with vigorous stirring. To this was added potassium acetate and the mixture was heated to reflux for 3 hrs. The reaction was cooled and concentrated providing a crude material that was subjected to RP-HPLC giving the product (505.6 mg, 32.2%) as a dark blue solid. MS *m/z* calculated for $C_{31}H_{36}ClN_2O_6S_2$ (M+H). Found 383.2 (M+H, ESI+): 631.2. Found 631.2 (M+H, ESI+).



4-(2-((*E*)-2-((*E*)-3-((*E*)-2-(1-(5-carboxypentyl)-1-methyl-9-sulfo-5,6-dihydro-1*H*-pyrrolo[3,2,1-*ij*]quinolin-2(4*H*)-ylidene)ethylidene)-5-chlorocyclohex-1-en-1-yl)vinyl)-1-methyl-9-sulfo-1,4,5,6-tetrahydropyrrolo[3,2,1-*ij*]quinolin-3-ium-1-yl)butane-1-sulfonate. To a suspension of hemicyanine (150.0 mg, 0.24 mmol), indolene (99.0 mg, 0.26 mmol) and potassium acetate (25.6 mg, 0.26 mmol) in ethanol (20 mL) was added triethylamine (36 µL, 0.26 mmol). The reaction was heated to reflux for 2.5 hrs, cooled and concentrated giving a dark residue that was dissolved in water and was purified by RP-HPLC, giving the product (150.0 mg, 68.8%) as a green solid. MS *m/z* calculated for $C_{44}H_{54}ClN_2O_{11}S_3$ (M+H): 917.3. Found 917.2 (M+H, ESI+).



4-(2-((*E*)-2-((*E*)-3-((*E*)-2-(1-(5-carboxypentyl)-1-methyl-9-sulfo-5,6-dihydro-1*H*-pyrrolo[3,2,1-*ij*]quinolin-2(4*H*)-ylidene)ethylidene)-5-(4-sulfophenoxy)cyclohex-1-en-1-yl)vinyl)-1-methyl-9-sulfo-1,4,5,6-tetrahydropyrrolo[3,2,1-*ij*]quinolin-3-ium-1-yl)butane-1-sulfonate (PBI 3920). A mixture of NaH (22.9 mg, 0.16 mmol) and 4-phenolsulfonic acid sodium salt (56.9 mg, 0.25 mmol) in DMF was added to a solution of chloro-dye (150.0 mg, 0.16 mmol) in DMF. The mixture was heated to 50°C for 1.5 hrs, cooled, diluted with water and was purified by RP-HPLC chromatography providing the title compound (62.5 mg, 36.3%) as a green solid. MS *m/z* calculated for $C_{50}H_{59}N_2O_{15}S_4$ (M+H): 1055.3. Found 1055.4 (M+H, ESI+).



4-(2-((*E*)-2-((*E*)-3-((*E*)-2-(1-(25-chloro-6-oxo-10,13,16,19-tetraoxa-7-azapentacosyl)-1-methyl-9-sulfo-5,6-dihydro-1*H*-pyrrolo[3,2,1-*ij*]quinolin-2(4*H*)-ylidene)ethylidene)-5-(4-sulfophenoxy)cyclohex-1-en-1-yl)vinyl)-1-methyl-9-sulfo-1,4,5,6-tetrahydropyrrolo[3,2,1-*ij*]quinolin-3-ium-1-yl)butane-1-sulfonate (PBI 3921). The above dye in free acid form (50.0 mg, 0.05 mmol), *N,N,N,N*-Tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (21.4 mg, 0.07 mmol) and diisopropylethylamine (33.0 µL, 0.2 mmol) were dissolved in DMF and allowed to react for 1 hr. To this was added 18-chloro-3,6,9,12-tetraoxaoctadecan-1-amine HCl (49.0 mg, 0.14 mol) and the reaction was stirred overnight in the dark. The crude reaction mixture was diluted with water and was purified by RP-HPLC giving the title compound (48.8 mg, 80.0%) as green solid. MS *m/z* calculated for $C_{64}H_{86}ClN_3O_{18}S_4$ (M+H):1348.5. Found 1348.5 (M+H, ESI+).

Table S1. Crystal data and structure refinement for cs1247.

Identification code	Promega1	
Empirical formula	$C_9H_{11}NO_3S•H_2O$	
Formula weight	231.26	
Temperature	100(2) K	
Wavelength	0.71073 Å	
Crystal system	Monoclinic	
Space group	P 21/c	
Unit cell dimensions	a = 7.9745(2) Å	□= 90°.
	b = 8.9150(2) Å	□= 95.322(2)°.
	c = 14.4539(3) Å	□ = 90°.
Volume	1023.14(4) $Å^3$	
Z	4	
Density (calculated)	1.501 Mg/m^3	
Absorption coefficient	0.310 mm^{-1}	
F(000)	488	
Crystal size	0.3342 x 0.1926 x 0.1018 mm^3	
Theta range for data collection	3.44 to 30.10°.	
Index ranges	-11<=h<=9, -12<=k<=12, -20<=l<=20	
Reflections collected	13327	
Independent reflections	3007 [R(int) = 0.0493]	
Completeness to theta = 30.10°	99.7 %	
Absorption correction	Gaussian	
Max. and min. transmission	0.971 and 0.928	
Refinement method	Full-matrix least-squares on F^2	
Data / restraints / parameters	3007 / 0 / 142	
Goodness-of-fit on F^2	1.099	
Final R indices [I>2sigma(I)]	R1 = 0.0368, wR2 = 0.1026	
R indices (all data)	R1 = 0.0476, wR2 = 0.1065	
Largest diff. peak and hole	0.487 and -0.395 $e.Å^{-3}$	

Table S2. Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters ($Å^2$x 10^3) for cs1247. U(eq) is defined as one third of the trace of the orthogonalized U^{ij} tensor.

	x	y	z	U(eq)
S(1)	2845(1)	-2896(1)	2148(1)	11(1)
O(1)	3630(1)	-2138(1)	2972(1)	15(1)
O(2)	1108(1)	-3356(1)	2272(1)	13(1)
O(3)	3842(1)	-4109(1)	1813(1)	18(1)
N(1)	3366(2)	2569(1)	975(1)	12(1)
C(1)	2951(2)	999(1)	736(1)	10(1)
C(2)	3101(2)	-43(1)	1455(1)	11(1)
C(3)	2659(2)	-1523(1)	1256(1)	10(1)
C(4)	2065(2)	-1945(1)	355(1)	13(1)
C(5)	1943(2)	-884(2)	-345(1)	13(1)
C(6)	2391(2)	619(1)	-173(1)	12(1)
C(7)	2285(2)	1742(2)	-961(1)	16(1)
C(8)	2386(2)	3355(2)	-617(1)	17(1)
C(9)	3769(2)	3504(2)	167(1)	15(1)
O(4)	507(1)	3704(1)	1571(1)	17(1)

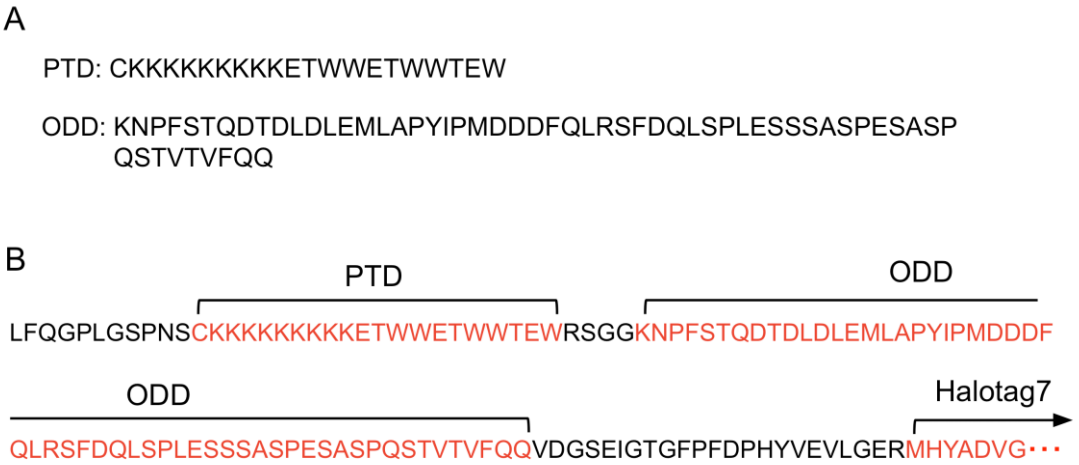


Figure S1. Amino acid sequence of POH protein components. (A) Amino acid sequences of PTD and ODD domains. The PTD sequence was termed PTD3 in our previous study [3]. The ODD amino acid sequences are corresponding to the residues of human HIF1-α 547-603. (B) Amino acid sequences of POH protein. POH fusion protein is composed of 406 amino acids including linker peptides between each domain. The theoretical molecular weight of POH protein is 46,002 Da according to the calculation of the sequences by GENETYX 7.0 (GENETYX CORP., Tokyo, Japan).

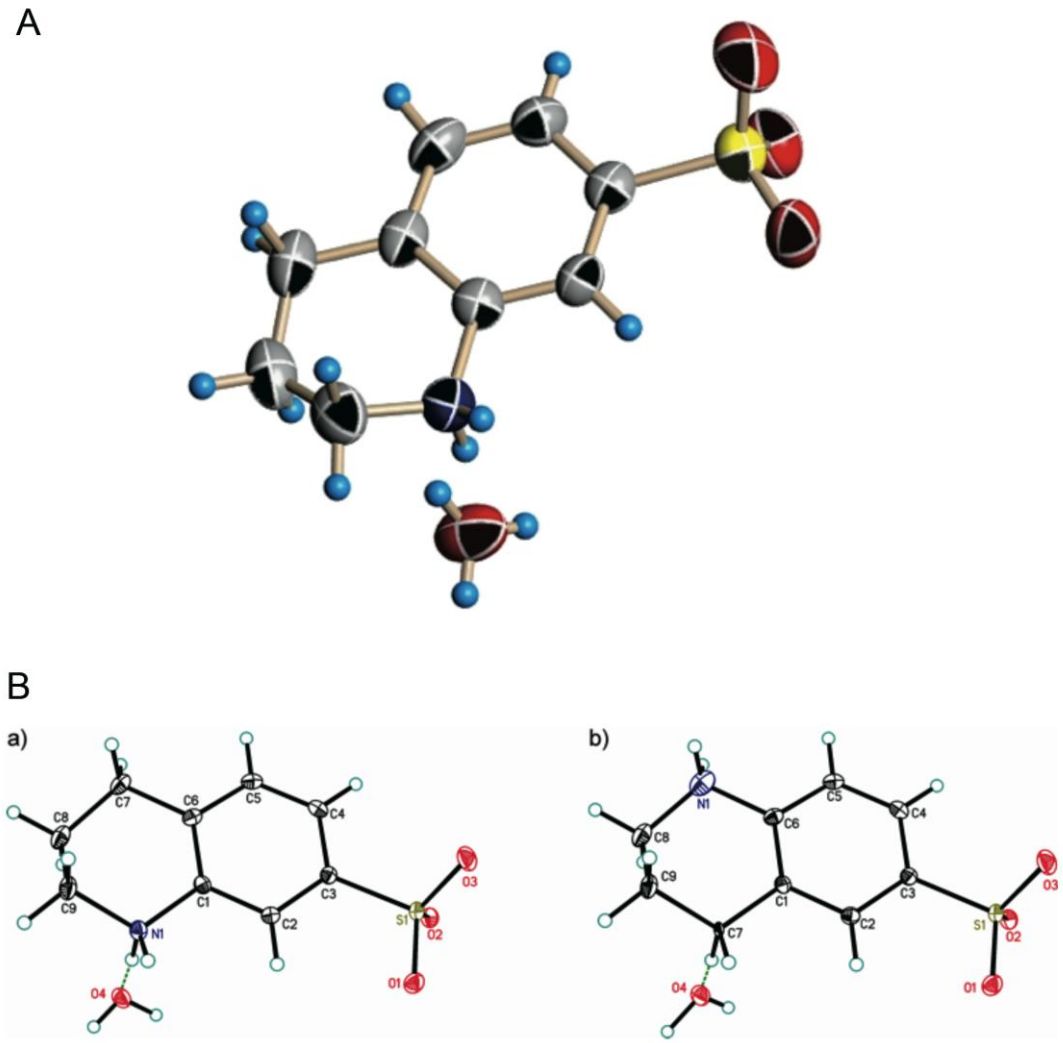


Figure S2. Crystal Structure of 1,2,3,4-tetrahydroquinolin-1-ium-7-sulfonate. (A) Molecular graphics of 1,2,3,4-tetrahydroquinolin-1-ium-7-sulfonate. (B) Displacement ellipsoid drawing (50%) for the (a) correct structural isomer and the (b) incorrect structural isomer. A colorless prism was cut into a thick plate and centered on the goniometer of an Oxford Diffraction Gemini diffractometer equipped with a Sapphire 3 CCD detector and operating with MoKα radiation. The data collection routine, unit cell refinement, and data processing were carried out with the program CrysAlisPro [4]. The Laue symmetry and systematic absences were consistent with the monoclinic space group $P2_1/c$. The structure was solved by direct methods and refined using SHELXTL NT [5]. The asymmetric unit of the structure comprises one zwiterionic hydrate. The final refinement model involved anisotropic displacement parameters for all non-hydrogen atoms. A riding model was used for the aromatic, alkyl, and ammonium hydrogen atoms.

Hydrogen atom positions for the water molecule were located from the residual electron density map and refined independently. SHELXTL NT was used for molecular graphics generation (Figure S2A) [5].

Because the crystals were submitted to identify the structural isomer present, extra care was used in assigning the nitrogen site. The nitrogen site was originally assigned based on the relative intensity of the Fourier peak in the structure solution. The nitrogen site was confirmed by comparing the relative sizes of the anisotropic displacement parameters. For the correct structural isomer, the equivalent isotropic thermal parameters for N1 and C7 were U_{eq}(N1) = 0.012(1) and U_{eq}(C7) = 0.016(1). For the incorrect structural isomer, the equivalent isotropic thermal parameters refined to a much less satisfactory U_{eq}(N1) = 0.0247(5) and U_{eq}(C7) = 0.0057(3) (Figure S2B). The hydrogen bonding between the ammonium group of the zwitterion and the water molecule provides the definitive conformation for the nitrogen position. In the correct structural isomer, a strong hydrogen bond forms between the ammonium group and the water molecule (N...O = 2.7073(16), <N-H...O = 170°. In the incorrect structure, this hydrogen bond is chemically unreasonable, since it would instead occur between a methylene group and water.

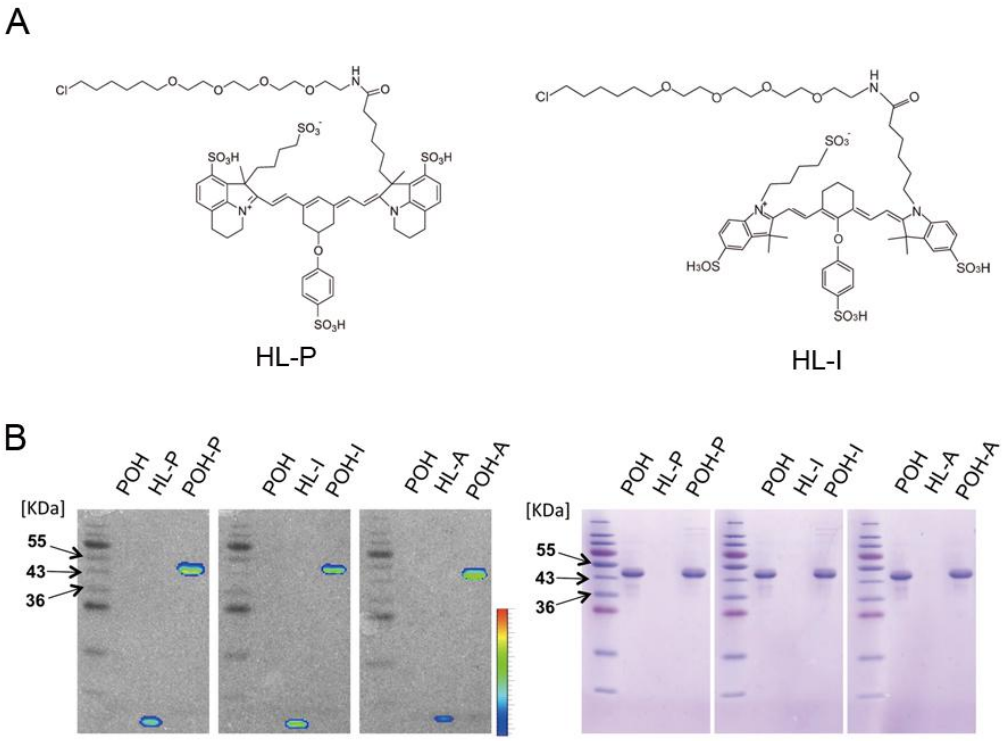


Figure S3. Construction of POH-Ns. (A) Chemical structure of HL-P and HL-I. (B) SDS-PAGE analysis of POH-Ns and HL-Ns. Probes resolved by SDS-PAGE were fluorescently scanned by IVIS (left panel) with excitation filter: 710 ± 15 nm, emission filter: 800 ± 10 nm for POH-P and POH-I or emission filter: 780 ± 10 nm for POH-A. SDS-PAGE gel was also stained with CCB (right panel).

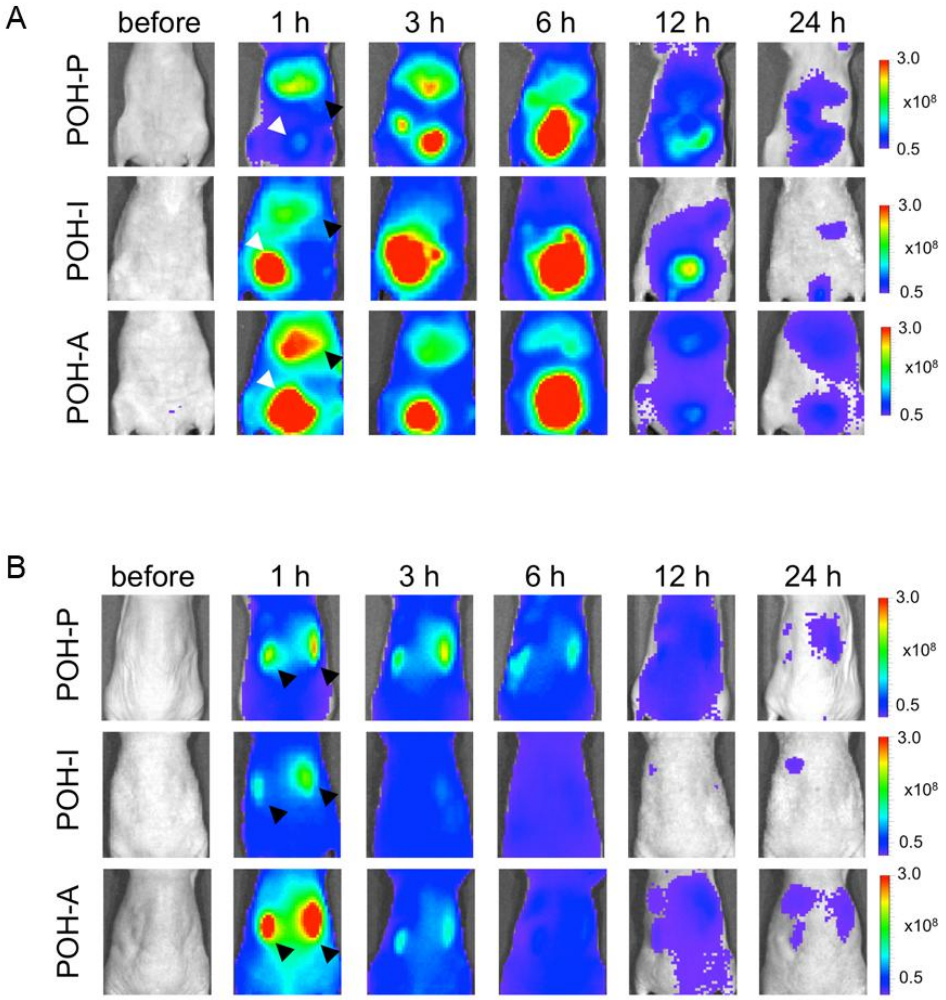


Figure S4. Time-course *in vivo* fluorescence images of POH-Ns for biodistribution analysis. (A) Representative sequential *in vivo* fluorescence images at ventral positions after POH-P (upper panel), POH-I (middle panel) and POH-A (bottom panel) injection. Black arrowheads indicate fluorescence from the liver. White arrowheads indicate fluorescence from the bladder due to urine excretion. (B) Representative sequential *in vivo* fluorescence images at dorsal positions after POH-P (upper panel), POH-I (middle panel) and POH-A (bottom panel) injection. Black arrowheads indicate fluorescence from the kidney.

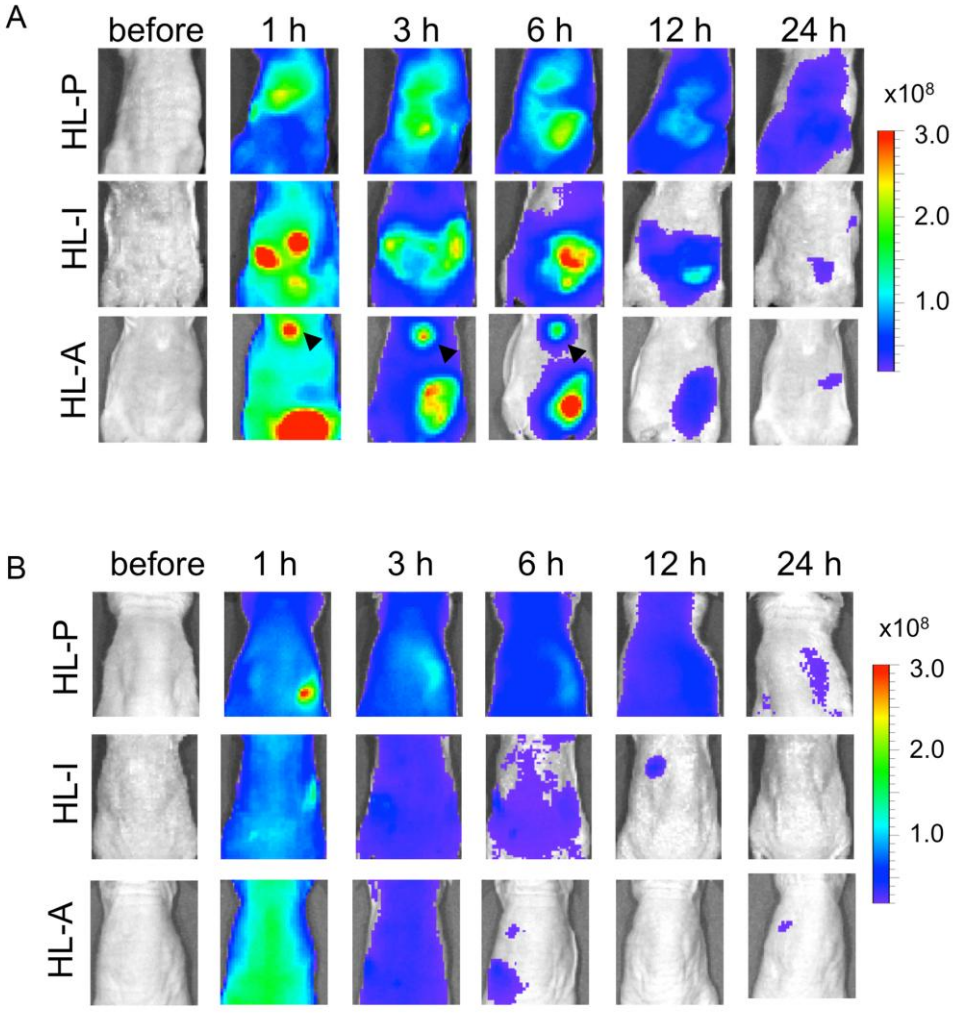


Figure S5. Time-course *in vivo* fluorescence images of HL-Ns for biodistribution analysis. (A) Representative sequential *in vivo* fluorescence images at ventral positions after HL-P (upper panel), HL-I (middle panel) and HL-A (bottom panel) injection. Black arrowheads indicate fluorescence from the galbladder. (B) Representative sequential *in vivo* fluorescence images at dorsal positions after POH-P (upper panel), POH-I (middle panel) and POH-A (bottom panel) injection.

References

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