Electronic Supplementary Information (ESI)

Silica supported tricarbocyanine based pH nanosensor with large Stokes shift and near infrared fluorescent response: performance in vitro and live cells

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General Information

All reagents used were of analytical grade and purchased from Sigma-Aldrich. They were used without further purification except otherwise stated. The solvents were dried and purified by standard methods prior to use. DAPI reagent was purchased from Invitrogen.

Nuclear Magnetic Resonance (NMR) spectra were measured with a Varian Bruker AC-200 and Bruker Avance II-500 spectrometers. Mass spectra were measured with a Bruker Daltonik microTOF mass spectrometer (ESI+, ESI-). IR spectra of the nanoparticles were recorded in KBr powders using PerkinElmer Spectrum BX FT-IR spectrometer with DRIFT by technique.

Particle size was assessed by field emission-scanning electron microscopy (FE-SEM), and dynamic light scattering (DLS) measurements. FE-SEM images were taken with a Zeiss Leo 982 Gemini microscope in the secondary-electron mode using an in-lens detector. DLS measurements were carried out in a Brookhaven BI-200 SM apparatus fitted with an avalanche photodiode detector and a He–Ne laser (wavelength 637 nm).

Corrected fluorescence emission spectra were obtained on a Cary Eclipse spectrophotometer equipped with two Czerny-Turner monochromators and a 15 W Xenon pulse lamp (pulse width: 2-3 us, power: 60-75 kW). Steady-state fluorescence measurements were carried out in a quartz 0.5 x 0.5 cm cell. The excitation wavelength was 685 nm, and the emitted light was collected at 90 with respect to the excitation beam.

To study the influence of pH in the samples, a solution of the dye or a suspension of the nanoparticles were titrated with aqueous solutions of NaOH (1M) or HCl (1M), under stirring. The pH values of the solutions were determined with a WTW GmbH 530 pH-meter.

Confocal laser scanning microscopy was performed using an Olympus Fluoview FV 1000 microscope with a UPLSAPO 60x 1.2 NA water immersion objective. Excitation and emission filters were as follows: excitation DAPI, 405 nm; emission DAPI, band pass (BP): 430–470 nm; excitation Probe, 637 nm; emission Probe, BP: 655-755 nm. Sequential scanning mode for image acquisition was always used.

Flow cytometry was performed with a Becton Dickinson FACSAriaII using a 633 nm laser for excitation, and a 660/20 nm BP emission filter. Data analysis was performed with WinMDI Version 2.9 software.

1. Synthesis of Cy-PIP (S4)

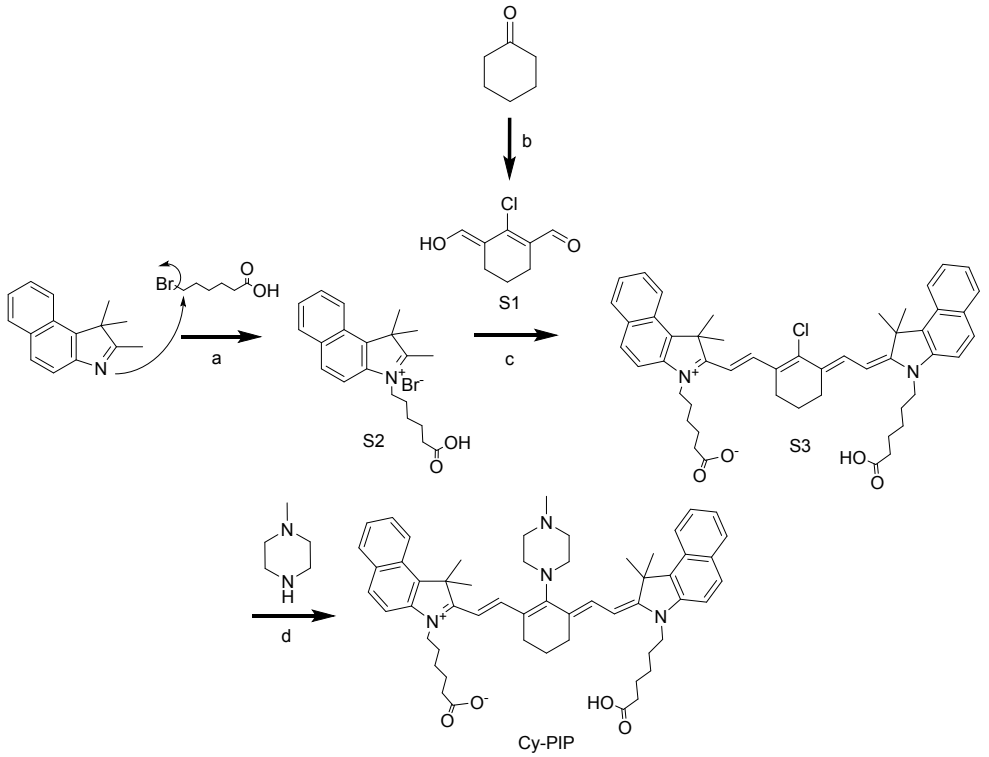


Figure S1 Reagents and conditions for the synthesis of Cy-PIP: (a) MeCN (anh), 150ºC, microwave (1 h), yield 95%, (b) 1) $POCl_3$, DMF, CH_2Cl_2, 0ºC, 30 min, 2) Addition of Ciclohexanone in 30 min at 0ºC, 3) Reflux, 3 h, room temperature, H_2O, -20ºC overnight, yield 46%; (c) 1) n-BuOH-Benzene (70:30), Reflux, 1h, 2) Py, S2, Reflux, 1h, then room temperature and low pressure evaporation with toluene, cold Et_2O, yield 49%, (d) DMF, TEA, overnight, in the dark, yield 62%.

2-chloro-3-(hydroxymethylene)cyclohex-1-ene-1-carbaldehyde (S1). Before starting the reaction, all the system was purged with argon gas. Keeping the temperature at 0 °C, a solution of phosphorus oxychloride (18.5 mL, 0.2 mol) in dichloromethane (17.5 ml, 0.2 mol) was added dropwise (30 minutes) from a round bottom flask through a stainless steel cannula to a two-necked round bottom flask containing a solution of anhydrous DMF (20.0 mL, 0.26 mol) and anhydrous dichloromethane (20.0 ml, 0.31 mol). The color of the solution turned yellow. At 0ºC, cyclohexanone (5.0 g, 5.3 ml, 0.05 mol) was added dropwise during 30 minutes (the reaction is highly exothermic) using a 10 ml glass syringe. The solution turned red. Once the addition of cyclohexanone was completed, the mixture was refluxed for 3 h. After this time, the reaction mixture was allowed to cool at room temperature (1 hour) and then cooled to -20ºC. The mixture was kept at that temperature overnight. The resulting yellow crystals were filtered and washed firstly with cooled water (10 ml) and then with diethyl ether (3 x 10 mL). The crystals were collected and dried under vacuum (room

temperature) to yield 3,97 g (46%). The product is unstable and has to be stored at -20ºC (for a period not longer than two weeks).

^{1}H NMR (200 MHz, DMSO-d_6) δ 2.35 (t, J = 6.1 Hz, 4H), 1.65 – 1.48 (m, 2H).

1,1,2-Trimethyl-3-(6-carboxy hexyl)- 1H-benzo[e]indol-3-ium-, Inner Salt (S2).

Anhydrous acetonitrile (6.0 mL), 1,1,2-trimethylbenzo[e]indol (0.96 g, 4.6 mmol), and 6-bromohexanoic acid (1.34 g, 6.8 mmol) were mixed in a 10 ml microwave reaction vessel and heated in a microwave oven for organic synthesis at 150ºC for 1 h. The reaction mixture was allowed to cool to room temperature and then cooled to -20ºC for 2 weeks, until precipitation occurred. The resulting grey crystals were filtered and washed with ether (3 x 10 mL). The crystals were collected and dried in vacuum to yield 1.75 g (95%). This product was used for the next reaction without further purification.

^{1}H NMR (200 MHz, $CDCl_3$) δ 8.19 – 7.89 (m, 3H), 7.87 – 7.51 (m, 3H), 4.59 (t, J = 7.3 Hz, 2H), 2.25 (t, J = 6.4 Hz, 2H), 2.02 – 1.83 (m, 2H), 1.75 (s, 6H), 1.68 – 1.55 (m, 2H), 1.47 (m, 2H).

Compound S3. A well dried two-necked round bottom flask equipped with a reflux condenser was charged with 1,1,2-trimethyl-3-(6-carboxy hexyl)- 1H-benzo[e]indol-3- ium-, inner salt **S2** (0.0698 g, 0.173 mmol) and 2-chloro-3-(hydroxymethylene)cyclohex-1-ene-1-carbaldehyde **S1** (0.0297 g, 0.173 mmol). Then, anhydrous *n*-butanol (9.4 ml) and anhydrous benzene (4.0 ml) were added using a 10 ml glass syringe. The system was purged with argon under stirring. The mixture was refluxed for 1 hour and the reaction was monitored by TLC. The mixture was allowed to cool to 50°C and 1,1,2-trimethyl-3-(6-carboxy hexyl)- 1H-benzo[e]indol-3- ium-, inner salt (0.0698 g, 0.173 mmol) and anhydrous pyridine (2.7 ml, 33.2 mmol) were added into the round bottom flask. The mixture was refluxed for another hour and then allowed to cool to room temperature. Pyridine was removed under reduced pressure in presence of toluene, the residue was washed with Et_2O to induce crystallisation. The green crude solid was purified by Sephadex column chromatography using methanol as eluent. Pure fractions were collected, evaporated and dried under vacuum, yielding a pure crystalline solid (87 mg, 49%).

^{1}H NMR (500 MHz, MeOD-d4) δ 8.55 (dd, J = 14.7, 3.0 Hz, 2H), 8.27 (d, J = 8.6 Hz, 2H), 8.09 – 7.95 (m, 4H), 7.65 (t, J = 8.9 Hz, 4H), 7.57 – 7.43 (m, 2H), 6.33 (dd, J = 14.6, 4.9 Hz, 2H), 4.31 (t, J = 6.7 Hz, 4H), 2.77 (t, J = 6.0 Hz, 4H), 2.34 (ddd, J = 15.6, 9.5, 5.3 Hz, 4H),

2.02 (s, 11H), 1.95 – 1.81 (m, 12H), 1.71 (dd, J = 14.5, 7.7 Hz, 4H), 1.54 (dd, J = 13.9, 7.3 Hz, 4H).

Cyanine piperazine (Cy-PIP, S4). Compound **S3** (95 mg) and N-methylpiperazine (25 ul, 0.2 mmol) were dissolved in 1 mL of anhydrous DMF, then triethylamine was added (123 µL, 0.9 mmol). The resulting solution was stirred overnight protected from light. The solvent was removed yielding an intense blue residue which was purified by Sephadex (LH-20, MeOH) column chromatography and RP-18 column chromatography (MeOH-H_2O 90:10) to afford the desired product as a blue solid (62.5 mg, 62%).

^{1}H NMR (500 MHz, MeOD-d_4) δ (ppm) 8.22 (d, J = 8.5 Hz, 2H), 7.99 – 7.94 (m, 6H), 7.61 (dd, J = 14.0, 7.1 Hz, 3H), 7.52 (d, J = 8.9 Hz, 2H), 7.46 – 7.40 (m, 2H), 6.08 (d, J = 13.7 Hz, 2H), 4.17 (t, J = 7.4 Hz, 4H), 3.79 (s, J = 4.7 Hz, 4H), 2.89 (s, 4H), 2.63 – 2.55 (m, 7H), 2.29 (t, J = 7.3 Hz, 4H), 2.02 (s, 12H), 1.93 – 1.85 (m, 6H), 1.72 (dt, J = 15.0, 7.3 Hz, 4H), 1.53 (t, J = 7.7 Hz, 4H).

^{13}C NMR (500 MHz, MeOD-d_4) δ (ppm) 178.77, 172.82, 142.59, 141.51, 133.88, 132.95, 132.38, 131.55, 131.07, 129.88, 129.63, 128.56, 126.38, 125.46, 123.17, 111.77, 97.88, 72.91, 57.72, 55.13, 51.44, 46.46, 44.51, 35.91, 28.87, 28.06, 27.62, 26.23, 26.16, 23.16.

HRMS (ESI+): calculated $[M+H]^+$ 847.51703; found: 847.51568.

2. NMR and HRMS Spectra characterization of Cy-PIP (S4)

For the complete NMR structural characterization, ^{1}H, ^{13}C, COSY and HSQC experiments had to be performed. For easy interpretation of the NMR signals we can consider the structure of the molecule shown in the Figure S1.

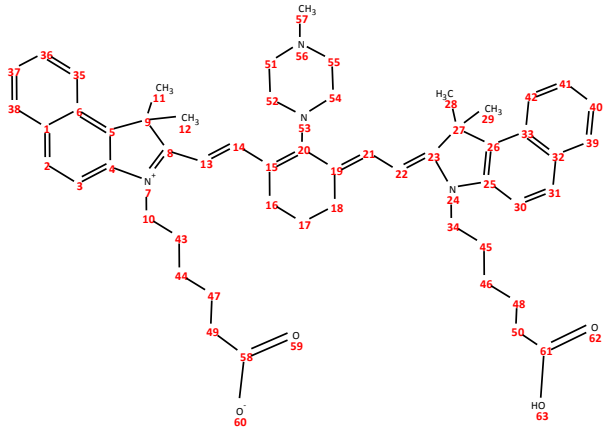


Figure S2. Chemical structure of Cy-PIP.

In the upper panel of Figure S3 we show the complete ^{1}H 500 MHz spectrum of Cy-PIP. Below, in the same figure, we indicate the expansions of the diagnostic signals: i) polymetinic chain, ii) methylene groups bonded to the indol and iii) piperazine. First, we have the signals at 7.96 ppm (Fig S3a) and 6.08 ppm (Fig S3b) that correspond to the 4 H of the polymetinic chain. The signal at 6.08 ppm is associated to 2H of the polymethinic chain (double bond, H_{13} and H_{22}). From HSQC experiments (not shown) we determined that the signal at 7.96 ppm is the result of 2 type of protons: the ones (H_{14} and H_{21}) of the polymethinic chain and 4H that belong to the aromatic ring.

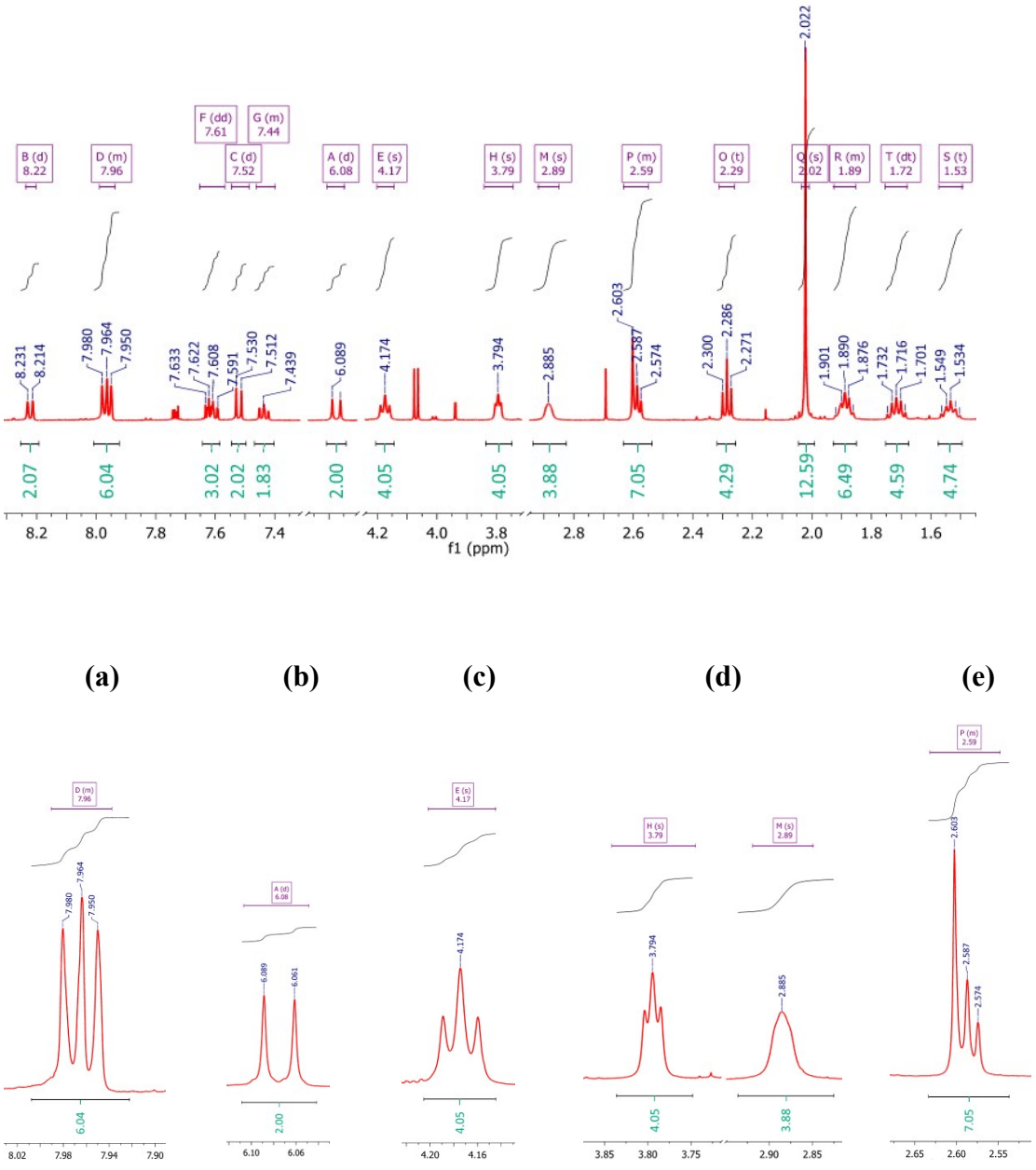


Figure S3. ^{1}H NMR Spectra of **S4**.

Also, we have the triplet at 4.17 ppm that belongs to the methylene groups bonded to the nitrogen of the indol structure (4H, 2 H_{10} and 2 H_{34}). The high chemical shift is due to the partial positive charge of the quaternary N of the indol structure. On the other hand, we have the signals at 3.79 (2 H_{52} and 2H_{54}) and 2.89 ppm (2 H_{51} and 2 H_{55}) that correspond to the methylene groups of the piperazine moiety. Finally, we have the asymmetric triplet at 2.59 ppm. From HSQC (not shown) we could determine that this signal is associated to two type of carbons, hence different type of protons. It is highly probable that the shape of the triplet is due to a convolution of a singlet (CH_3 of the piperazine, 3 H_{57}) and a triplet (2 methylene groups of the central ring, 2 H_{16} and 2 H_{18}).

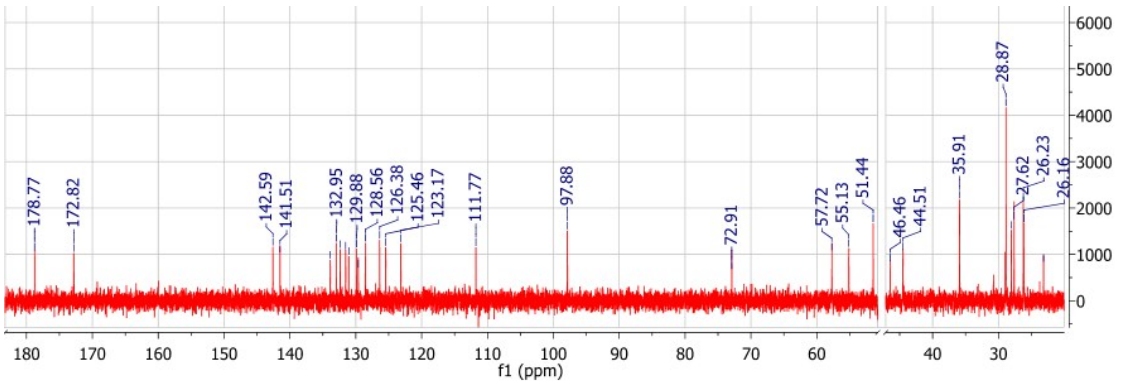


Figure S4. ^{13}C NMR Spectra of compound **S4**.

Qualitative Compound Report

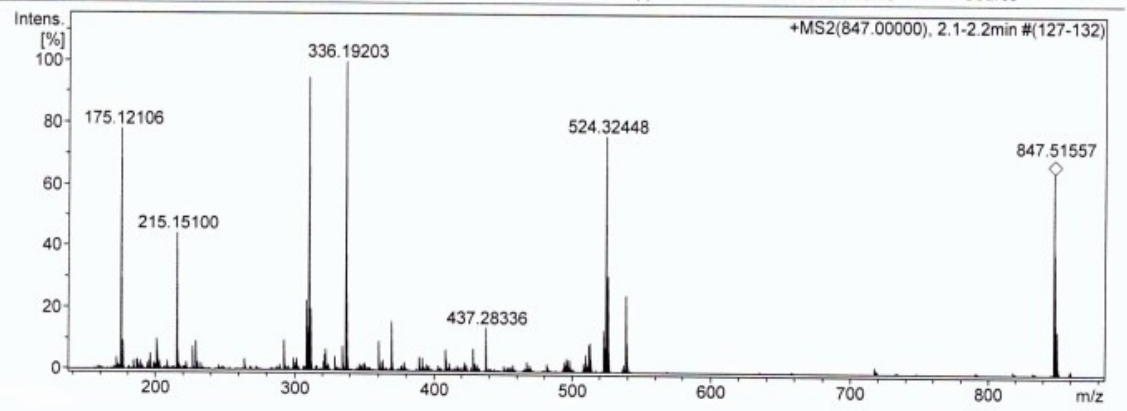
Analysis Info

Analysis Name	D:\Data\ggc\E-80.d	Acquisition Date	1/9/2015 12:37:08 PM
Method	tune_wide.m	Operator	Gabriel Cases
Sample Name	E-80	Instrument	micrOTOF-Q II
Comment	Sv: MeOH Carla Spagnuolo		

UMYMFOR CONICET FCEN - UBA

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	100 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	1200 m/z	Set Collision Cell RF	300.0 Vpp	Set Divert Valve	Source



Meas. m/z	#	Formula	m/z	err [ppm]	Mean err [ppm]	e⁻ Conf	N-Rule	mSigma
175.12106	1	C 8 H 17 N O 3	175.12029	-4.36	-4.33	odd	ok	18.5
215.15100	1	C 11 H 21 N O 3	215.15159	2.74	1.10	odd	ok	16.8
	2	C 9 H 19 N 4 O 2	215.15025	-3.50	-5.17	even	ok	21.9
310.17668	1	C 18 H 22 N 4 O	310.17881	6.86	7.19	odd	ok	5.1
	2	C 15 H 24 N 3 O 4	310.17613	-1.78	-1.44	even	ok	22.0
336.19203	1	C 20 H 24 N 4 O	336.19446	7.24	7.27	odd	ok	15.8
	2	C 17 H 26 N 3 O 4	336.19178	-0.73	-0.68	even	ok	33.9
369.29658	1	C 20 H 39 N 3 O 3	369.29859	5.45	5.91	odd	ok	42.4
	2	C 22 H 41 O 4	369.29994	9.09	9.56	even	ok	49.1
428.25484	1	C 29 H 34 N O 2	428.25841	8.33	8.49	even	ok	32.5
	2	C 27 H 32 N 4 O	428.25706	5.19	5.34	odd	ok	33.0
512.32004	1	C 37 H 40 N 2	512.31860	-2.82	-3.29	odd	ok	42.4
524.32448	1	C 34 H 42 N 3 O 2	524.32715	5.09	4.79	even	ok	14.5
538.33761	1	C 35 H 44 N 3 O 2	538.34280	9.65	7.73	even	ok	41.3
847.51557	1	C 55 H 67 N 4 O 4	847.51568	0.13	0.56	even	ok	15.4

Figure S5. ESI HRMS Report of compound **S4**

3. Synthesis of APTES@SiO_2 nanoparticles

APTES@SiO_2 nanoparticles were prepared by a modification of the Stöber method. Briefly, 8.6 g (4.1 mmol) of TEOS were rapidly added to a solution containing 157.5 g of absolute ethanol, 6.7 g of NH_4OH concentrated solution and 3.7 g of MilliQ Water. After 5 hours of reaction under mild stirring, a solution of 0.3 ml aminopropyltriethoxysilane (APTES) in 5 ml of absolute ethanol was added to the reaction mixture. The reaction was stirred at room temperature for 18 hours, and then the mixture was centrifuged (7200 rpm, 30 minutes). The nanoparticles were washed with ethanol and deionized water by cycles of sonication and centrifugation, until the pH of the supernatant reached 7. After drying at 60°C under vacuum for 16 hours, the product was obtained as a white powder.

Synthesis of Cy-PIP@SiO_2 nanoparticles

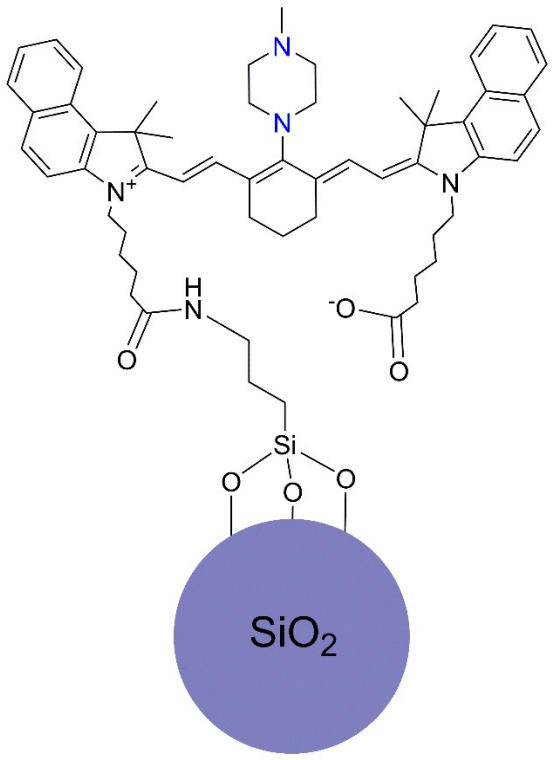


Figure S6 Schematic representation of Cy-PIP@SiO_2 nanoparticles.

100 µl of an anhydrous DMF (1.0 ml) solution of 1,1-carbonildiimidazol (11.6 mg) were added into a solution of Cy-PIP dye (4.2 mg) in anhydrous DMF (265 µl). The system was purged with nitrogen and stirred for 1 hour until activation of the carboxylic acid groups of the molecule had occurred. Then, the liquid mixture was added to a well dispersed and stable

suspension of 20.9 mg of APTES@SiO_2 in 200 µl of DMF. The mixture was allowed to react overnight, protected from the light. Then, any remaining reactants were removed by cycles of centrifugation-resuspension, until the supernatant showed no fluorescence emission (λ_{exc} = 650 nm).

4. FE-SEM and DLS

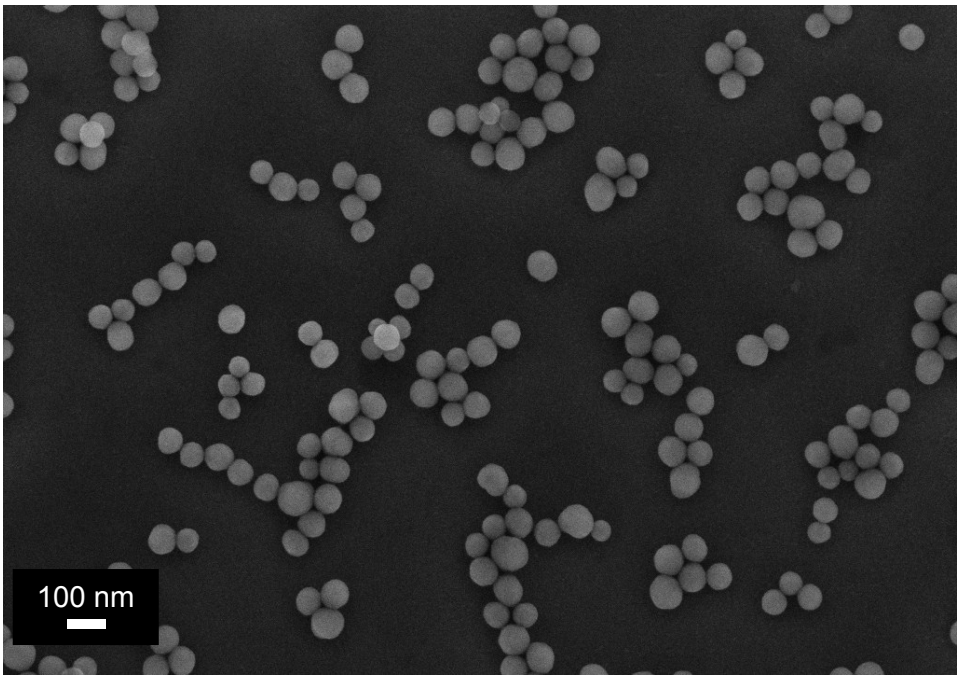


Figure S7 FE-SEM micrograph of Cy-PIP@SiO_2 NP.

The size determined by treating the FESEM image with Image J Software was **88.5 nm (SD = 12.6 nm; N = 150 NP)**.

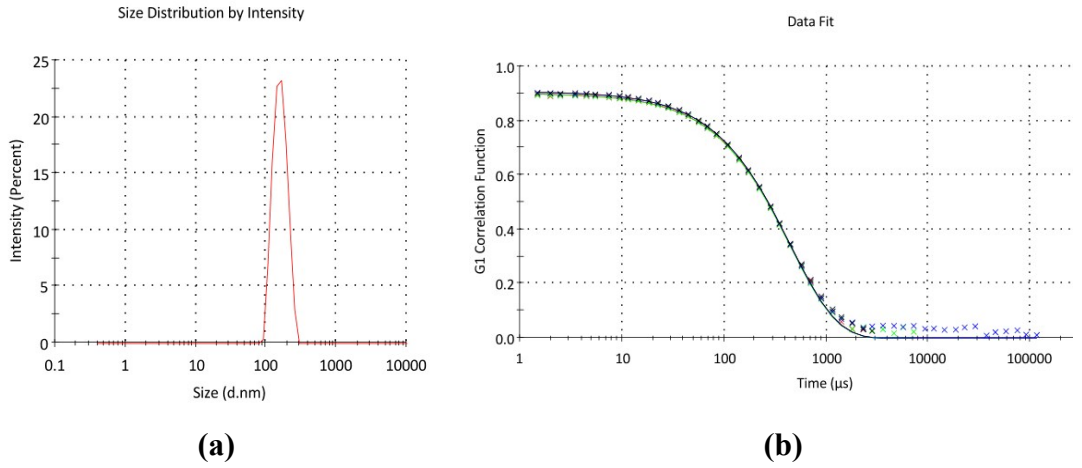


Figure S8 DLS measurements of a Cy-PIP@SiO_2 NP water dispersion at room temperature (25ºC). A size of 151.5 ± 0.5 nm was calculated (4 independent measuments). (a) size distribution by intensity. The fit to the correlation function (4 measurements) is shown in (b).

5. FTIR spectroscopy

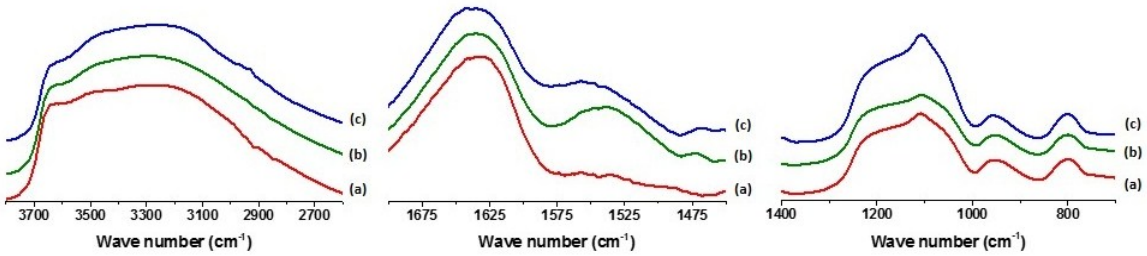


Figure S9 FTIR Spectra (Diffuse Reflectance Infrared Fourier Transform Spectroscopy, DRIFTS): (a) Bare SiO_2 nanoparticles, (b) APTES@SiO_2 and (c) Cy-PIP@SiO_2.

The FTIR of the SiO_2, APTES@SiO_2 and Cy-PIP@SiO_2 nanoparticles showed an intense band at 3600-3100 cm^{-1}, assigned to O-H stretching vibrations. In all cases, FTIR spectra show a characteristic large band at 1000-1100 cm^{-1} associated to typical Si-O and O-Si-O vibrations. Non-calcined SiO_2 sol-gel films exhibit a strong O-H stretching peak at 3400 cm^{-1} where hydrogen bonded absorbed water has an important role [1,2]. Also, the 3000-3800 cm^{-1} region includes O-H stretching from silanols (isolated SiOH (3750 cm^{-1}), geminal SiOH (3742 cm^{-1}) and vicinal SiOH (3720-3520 cm^{-1}) [1,2]). On the other hand, the characteristic

bands of aliphatic $\nu_{C\text{-}H}$ at 2900-2800 cm^{-1} are hardly distinguished [2,3] for anchored amino propyl groups and immobilized Cy-PiP moieties (Fig. S8b and Fig. S8c, respectively).

Protonated primary amines ($–NRH_2^+$) have medium-to-strong absorptions near 1600 cm^{-1} and 1500 cm^{-1} due to asymmetric and symmetric deformation vibrations [3]. These bands are clearly seen in figure S8b, between 1580 and 1480 cm^{-1}. In figure S8c, these $–NRH_2^+$ bands appear, but they are overlapped with a band associated with a characteristic, strong absorption at 1570-1515 cm^{-1} of secondary amides in the solid phase (amide II band, N-H deformation and C-N stretching vibrations) [3]. The carbonyl group C-O stretching vibrations (amide I band) cannot be distinguished because of the strong and wide water vibration band near 1643 cm^{-1}. Neither the $–NRH_2^+$ nor the amide II bands are present in figure S8a, the bare SiO_2 nanoparticles used as a control.

6. Photophysical characterisation of Cy-PIP

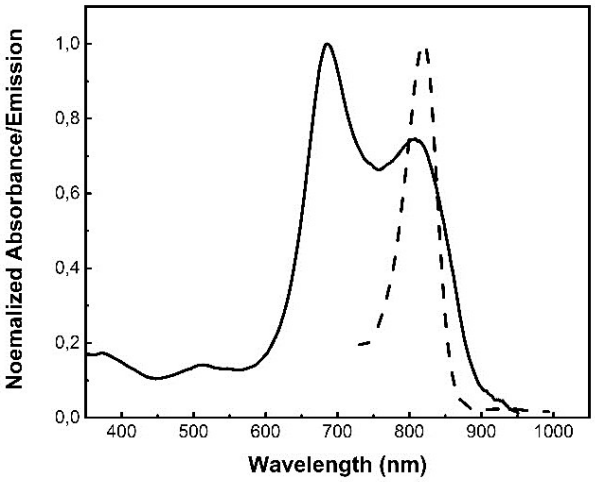


Figure S10 Normalized absorption (solid line) and emission (dashed line) spectra of Cy-PIP dye solution (5 μM) in phosphate buffer 10 mM pH 7.30; (λ_{exc} = 685 nm; slit = 20 nm).

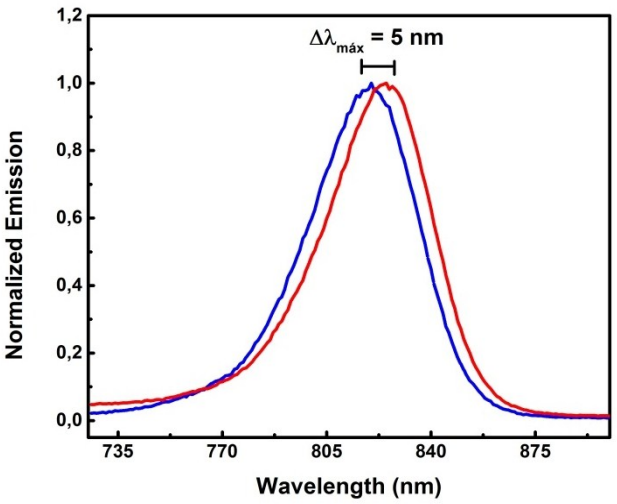


Figure S11 Comparison between normalized emission spectra of free Cy-PIP dye (M4, blue) and Cy-PIP@SiO_2 NP (red) in PBS pH 7.30 (λ_{exc} = 685 nm; slit = 20 nm).

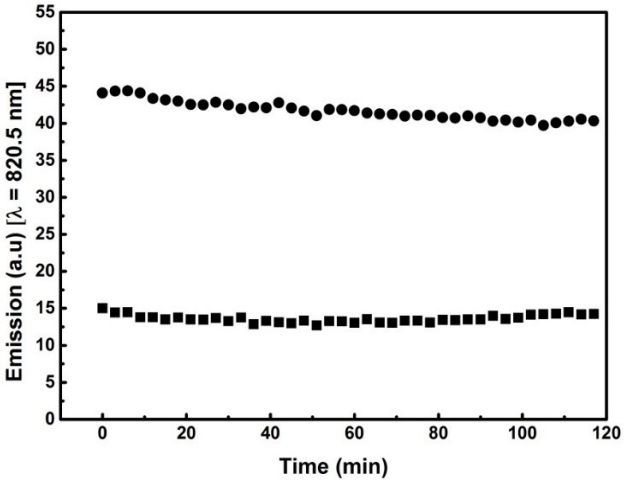


Figure S12 Time dependence of free Cy-PIP dye fluorescence at 820.5 nm in phosphate buffer 10 mM pH 5.1 (■) and pH 9.5 (●) (λ_{exc} = 685 nm; slit = 20 nm).

7. Cell culture and imaging

Human cervical carcinoma HeLa cells (ATCC-CCL-2) were grown in Dulbecco's modified Eagle's medium (GIBCO) supplemented with penicillin, streptomycin and 10% FBS at 37°C in 5% CO_2. Cells were plated at 1×10^5 cells/well onto 12 mm glass coverslips in 24 well plates (for microscopy experiments) or at 2.5×10^6 cells in 60 mm dishes (for flow cytometry analysis) 24 hours prior to treatment. To evaluate the uptake and internalization of the nanoprobe, cells were treated for 1 hour with serial dilutions of Cy-PIP@SiO2 nanoparticles suspensions, washed with PBS and incubated for 240 min at 37°C in culture media. Cells were fixed with 5% w/v paraformaldehyde and analyzed by confocal microscopy. Although

at the mg/ml range cells showed membrane blebbing/protrusions and detachment, at 2 ug/ml no apparent biological toxicity was observed maintaining a robust fluorescence signal within the experimental time frame.

8. Flow cytometry

Flow cytometry was performed with a Becton Dickinson FACSAriaII using a 633 nm laser for excitation, and a 660/20 nm BP emission filter. Data analysis was performed with WinMDI Version 2.9 software. Forward-scattered (FSC) and side-scattered (SSC) light graphs show no differences in size, shape and structure of the cells during the exposure to Cy-PIP@SiO_2 nanoparticles when compared to HeLa control cells.[4] This is consistent with the confocal microscope observations regarding the absence biological toxicity of the NPs at the working concentration**.** Histograms were performed using a gate for positive events for probe signal taking into account basal auto-fluorescence of control cells (cells not treated with NP). A geometric mean was calculated for each histogram using the WinMDI. In each histogram a gate was used to determine events with high fluorescent signal including approximately 20% of the events at the initial moment (0 min). For each internalization period the percentage of events inside this gate was measured.

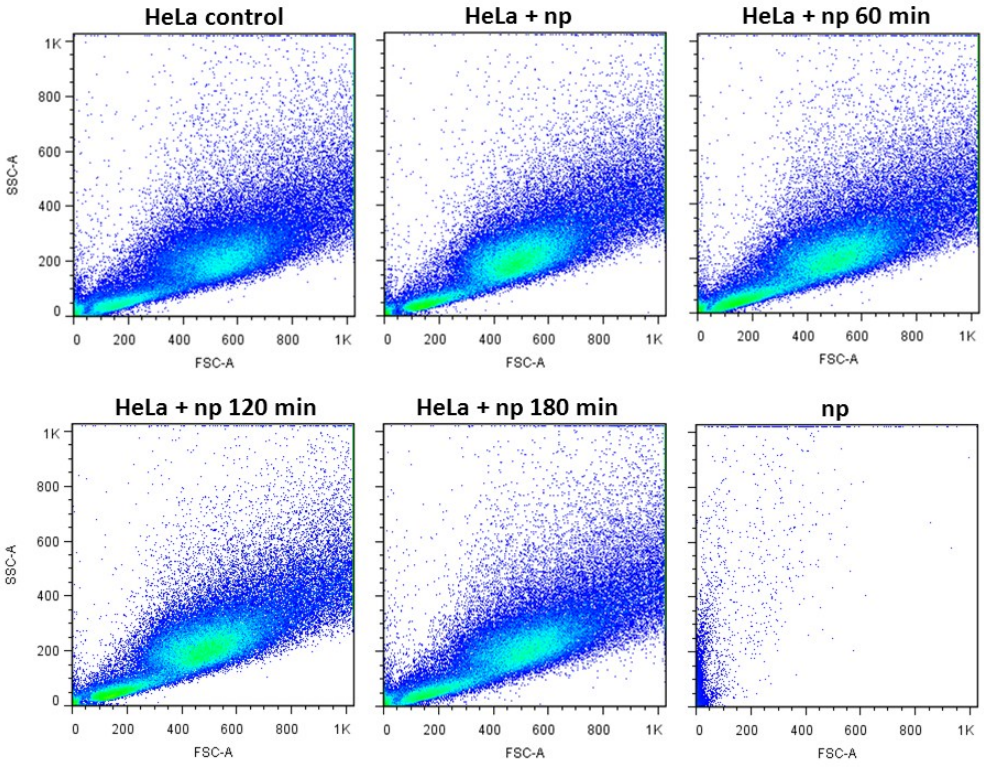


Figure S13 Flow cytometry graphs plotting forward-scattered (FSC) light (proportional to cell-surface area or size) vs side-scattered (SSC) light (proportional to cell granularity or internal complexity).

References

[1] (a). Almeida, R.M ; Pantano, C.G. Structural investigation of silica gel films by infrared spectroscopy *J. Appl. Phys*. **1990,** *68,* 4225; (b) Sacks, M.D. ; Tseng, T.Y. Preparation of SiO_2 Glass from Model Powder Compacts: II, Sintering *J. Am. Ceram. Soc* **1984,** *67,* 532; (c) Burnean, A.; Barrès, O.; Galleas, J.P.; Lavalley, J.C. Comparative study of the surface hydroxyl groups of fumed and precipitated silicas. 2. Characterization by infrared spectroscopy of the interactions with water *Langmuir* **1990,** *6,* 1364.

[2] Marchena, M.H.; Granada, M.; Bordoni, A.V.; Joselevich, M.; Troiani, H.; Williams, F.J.; Wolosiuk, A. Organized thiol functional groups in mesoporous core shell colloids J. *Solid State Chem.* **2012,** *187,* 97-102.

[3] Socrates, G. Infrared and Raman Characteristic Group Frequencies (3rd Edn). John Wiley and Sons: Chichester, **2001**.

[4] H. M. Shapiro, *Practical Flow Cytometry*, Wiley, **2005**.