

Supporting Information

Protein-Sized Bright Fluorogenic Nanoparticles Based on Cross-linked Calixarene Micelles with Cyanine Corona

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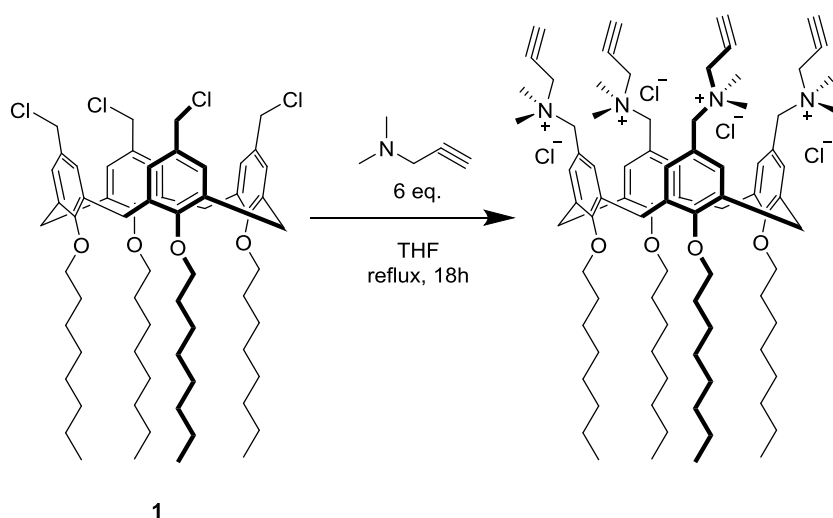
Materials and methods

Synthesis of organic building blocks (compounds)

All starting materials for synthesis were purchased from Alfa Aesar and Sigma Aldrich or TCI Europe and used as received unless stated otherwise. NMR spectra were recorded on a BrukerAvance III 400 MHz spectrometer. Mass spectra were obtained using an Agilent Q-TOF 6520 mass spectrometer. MilliQ-water (Millipore) was used in all experiments.

Synthesis of amphiphilic calixarene derivative (CX8TP)

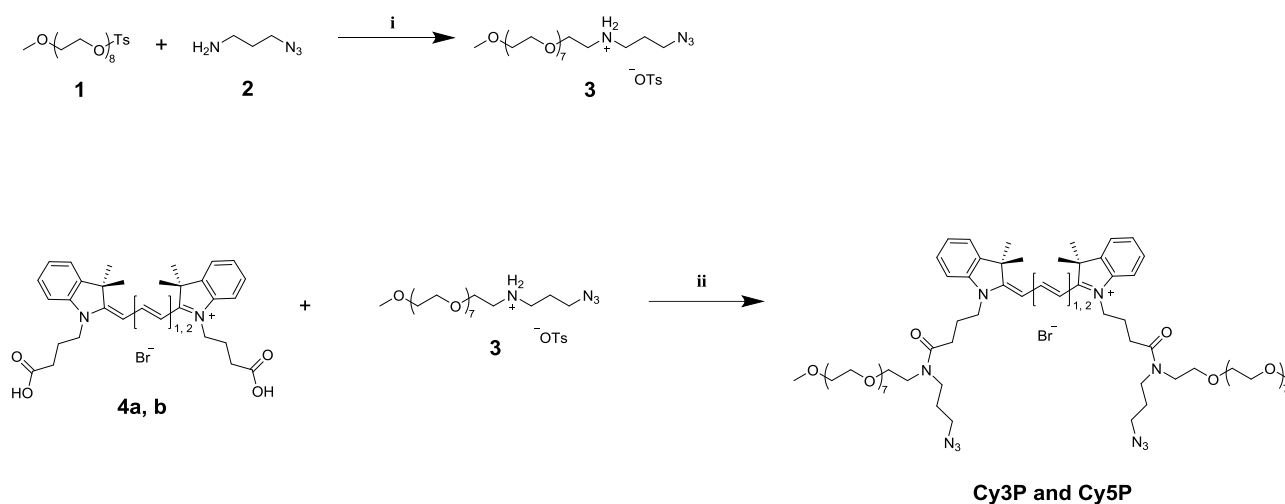
5,11,17,23-Tetra(N,N-dimethyl-N-(propargyl)ammonium)methylene-25,26,27,28-tetraoctyloxycalix[4]arene tetrachloride.



A solution of 3-dimethylamino-1-propyne (6 eq., 112 mg, 145 μ L, 1.35 mmol) in THF (5 mL) was added dropwise to a solution of 5,11,17,23-Tetrachloromethyl-25,26,27,28-tetraoctyloxycalix[4]arene (1 eq., 240 mg, 0.225 mmol) in dry THF (10 mL) at r.t. Then the reaction mixture was refluxed for 18h. After cooling, the formed precipitate (calixarene **CX8TP**) was filtered off and washed with dry THF (10 mL) and dry diethyl ether (10 mL). The precipitate was dried under vacuum (0.05 mm Hg, 20 $^{\circ}$ C, 4 h) to give a desired product as a pale-yellow crystalline compound. Yield 239mg, 76%.

^1H NMR (400 MHz, Methanol- d_4) δ 7.07 (s, 8H), 4.58 (d, J = 13.3 Hz, 4H), 4.57 (s, 8H), 4.23 (s, 8H), 4.05 (t, J = 7.3 Hz, 8H), 3.68 (t, J = 2.3 Hz, 4H), 3.46 (d, J = 13.4 Hz, 4H), 3.11 (s, 24H), 2.05 (p, J = 7.3 Hz, 8H), 1.55 – 1.38 (m, 40H), 0.98 (t, J = 6.7 Hz, 12H). **^{13}C NMR (101 MHz, MeOD)** δ 159.98, 137.12, 134.61, 122.50, 83.46, 76.95, 72.76, 68.35, 54.51, 50.26, 33.23, 31.71, 31.45, 31.19, 30.86, 27.66, 23.83, 14.49. **HRMS (ESI) m/z :** $[\text{M}]^{4+}/4$ calcd for $\text{C}_{84}\text{H}_{128}\text{N}_4\text{O}_4^{4+}$, 314.2478; found, 314.2487; $[\text{M}+\text{Cl}]^{3+}/3$ calcd for $\text{C}_{84}\text{H}_{128}\text{ClN}_4\text{O}_4^{3+}$, 430.6536; found, 430.6538.

Synthesis of bi-functional cyanine fluorescent cross-linkers



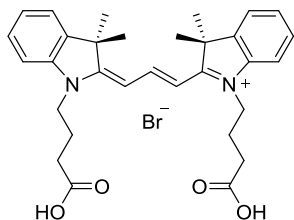
i) EtOH, reflux, 24h (75%); ii) HBTU, HOBt, DIPEA, DMF, r.t., 24h (74%).

N-(3-azidopropyl)-2,5,8,11,14,17,20,23-octaoxapentacosan-25-aminium 4-methylbenzenesulfonate (3). Octaethylene glycol monomethyl ether tosylate (1 eq., 1.79 g, 3.33 mmol) (and 3-azidopropyl-1-amine (3 eq., 1 g, 9.99 mmol) were dissolved in anhydrous ethanol (20 mL).

Reaction mixture was stirred under reflux for 24 h. After cooling down to room temperature solvent was removed under reduced pressure. The product was purified by gradient column chromatography (SiO₂, DCM/MeOH, 95:5 to 90:10), which furnished 1.595 g (yield 75%) of a title compound **3**. **¹H NMR (400 MHz, Methanol-*d*₄)** δ 7.76 (d, *J* = 8.0 Hz, 2H), 7.29 (d, *J* = 7.9 Hz, 2H), 3.79 (t, *J* = 5.1 Hz, 2H), 3.72 – 3.67 (m, 26H), 3.62 – 3.57 (m, 2H), 3.52 (t, *J* = 6.5 Hz, 2H), 3.41 (s, 3H), 3.24 (t, *J* = 5.1 Hz, 2H), 3.18 – 3.09 (m, 2H), 2.42 (s, 3H), 1.98 (p, *J* = 6.8 Hz, 2H). **¹³C NMR (101 MHz, Methanol-*d*₄)** δ 143.65, 141.63, 129.81, 126.95, 72.92, 71.59, 71.56, 71.54, 71.53, 71.52, 71.49, 71.42, 71.31, 71.25, 71.24, 71.08, 67.28, 59.09, 49.72, 48.73, 46.59, 27.08, 21.32. **HRMS ESI (m/z):** [M]⁺calcd. for C₂₀H₄₃N₄O₈, 467.3075; found, 467.3083.

1-(3-carboxypropyl)-2,3,3-trimethyl-3H-indol-1-ium bromide (5). 1-(4-ethoxy-4-oxobutyl)-2,3,3-trimethyl-3H-indol-1-ium bromide (1 eq., 4 g, 11.3 mmol) was placed in a reaction flask. 20 mL of 30% aq. solution of hydrobromic acid were added. Obtained mixture was stirred at 110 °C for 4 hours. After cooling to room temperature, solvent was removed under reduced pressure. To the residue 50 mL of acetonitrile were added and a white precipitate was removed by filtration, washed several times with acetonitrile, then once with diethyl ether. This furnished 2.91 g (yield 79%) of the title compound **5** as a white solid. **¹H NMR (400 MHz, DMSO-*d*₆)** δ 12.21 (s, 1H), 8.08 – 7.98 (m, 1H), 7.90 – 7.80 (m, 1H), 7.68 – 7.57 (m, 2H), 4.54 – 4.45 (m, 2H), 2.86 (s, 3H), 2.54 (t, *J* = 7.0 Hz, 2H), 2.07 (p, *J* = 7.3 Hz, 2H), 1.55 (s, 6H). **¹³C NMR (101 MHz, DMSO-*d*₆)** δ 196.80, 173.64, 141.85, 141.15, 129.32, 128.86, 123.48, 115.27, 54.17, 46.97, 30.32, 22.47, 21.98, 14.05. **HRMS (m/z):** [M]⁺calcd. for C₁₅H₂₀NO₂, 246.1489; found, 246.1496.

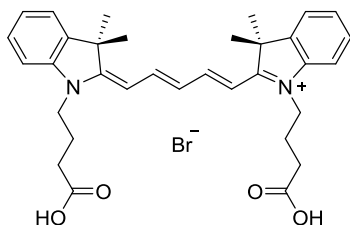
Cyanine 3 bisacid (4a). Compound **5** (1 eq., 2 g, 6.13 mmol) was placed in a reaction flask.



Anhydrous pyridine (40 mL) was added via syringe. Obtained mixture was preheated to 110 °C until complete dissolving of indoleninium salt, then triethylorthoformate (1.5 eq., 1.53 mL, 9.2 mmol) was quickly added dropwise to the boiling solution of indoleninium salt using syringe. The reaction mixture was stirred under reflux for 4 h. Solvent was removed under reduced pressure. The residue was dissolved in DCM (100 mL), washed with

1M aq. solution of hydrochloric acid (3 × 100 mL), and once with brine, dried over sodium sulphate. After solvent evaporation, the product was purified by gradient column chromatography (SiO₂, DCM/MeOH/HCOOH, 9:1:0 to 9:1:0.1), which furnished 2.46 g (yield 69%) of the title compound **4a** as a red solid. ¹H NMR (400 MHz, DMSO-*d*₆) δ 12.50 (br s, 2H), 8.36 (t, *J* = 13.4 Hz, 1H), 7.64 (d, *J* = 7.4 Hz, 2H), 7.50 (d, *J* = 8.0 Hz, 2H), 7.45 (td, *J* = 8.0, 1.2 Hz, 2H), 7.30 (t, *J* = 7.4 Hz, 2H), 6.57 (d, *J* = 13.4 Hz, 2H), 4.15 (t, *J* = 7.7 Hz, 4H), 2.42 (t, *J* = 7.1 Hz, 4H), 1.96 (p, *J* = 7.3 Hz, 4H), 1.70 (s, 12H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 173.95, 173.75, 149.93, 141.86, 140.61, 128.59, 125.17, 122.49, 111.36, 102.68, 48.89, 43.18, 30.78, 27.41, 22.41. HRMS (*m/z*): [*M*]⁺ calcd. for C₃₁H₃₇N₂O₄, 501.2748; found, 501.2756.

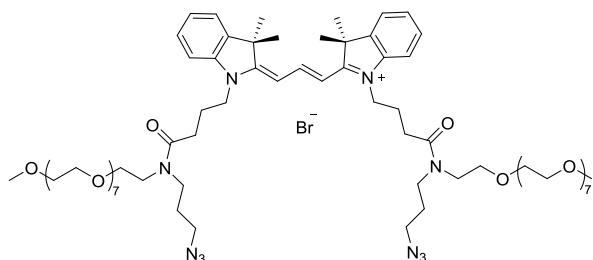
Cyanine 5 bisacid (4b). Compound **5** (1 eq., 2 g, 6.13 mmol) was placed in a reaction flask.



Anhydrous pyridine (40 mL) was added via syringe. Obtained mixture was preheated to 110 °C until complete dissolving of indoleninium salt, then 1,1,3,3-tetramethoxypropane (1.5 eq., 1.53 mL, 9.2 mmol) was quickly added dropwise to the boiling solution of indoleninium salt using syringe. The reaction mixture was stirred under reflux for 4 hours. Solvent was removed under reduced pressure. The residue was

redissolved in DCM (100 mL), washed with 1M aq. solution of hydrochloric acid (3 × 100 mL), and once with brine, dried over sodium sulphate. After solvent evaporation, the product was purified by gradient column chromatography (SiO₂, DCM/MeOH/HCOOH, 9:1:0 to 9:1:0.1), which furnished 2.64 g (yield 71%) of the title compound **4b** as a dark blue solid. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.35 (t, *J* = 13.1 Hz, 2H), 7.61 (d, *J* = 7.5 Hz, 2H), 7.46 – 7.33 (m, 4H), 7.24 (td, *J* = 7.1, 1.7 Hz, 2H), 6.54 (t, *J* = 12.3 Hz, 1H), 6.37 (d, *J* = 13.8 Hz, 2H), 4.11 (t, *J* = 7.7 Hz, 4H), 2.42 (t, *J* = 7.0 Hz, 4H), 1.95 – 1.83 (m, 4H), 1.68 (s, 12H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 173.87, 172.70, 154.14, 141.93, 141.07, 128.36, 125.50, 124.64, 122.40, 110.89, 103.14, 48.89, 42.76, 30.49, 27.10, 22.19. HRMS (*m/z*): [*M*]⁺ calcd. for C₃₃H₃₉N₂O₄, 527.2904; found, 527.2915.

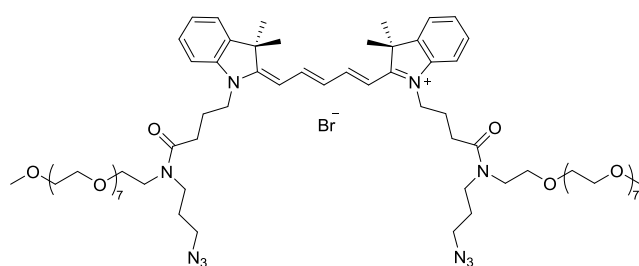
“Clickable” cyanine 3 cross-linker Cy3L. Cy3 bis-acid **4a** (1 eq., 100 mg, 0.2 mmol), HBTU (2.2 eq.,



166 mg, 0.439 mmol), and HOBt (3 eq., 81 mg, 0.599 mmol) were placed in a reaction flask. Anhydrous DMF (3 mL) and DIPEA (10 eq., 330 μL, 2 mmol) were added via syringe. After stirring for 30 min, compound **3** (1.9 eq., 242 mg, 0.38 mmol) was added dropwise.

The reaction mixture was set to stir at r.t. for 24 h under argon atmosphere. Solvent was removed under reduced pressure. The residue was redissolved in 50 mL of DCM and washed with brine (3 × 100 mL) to remove traces of DMF, dried over sodium sulphate, filtered and the filtrate was evaporated to dryness in vacuum. The product was purified by gradient column chromatography (SiO₂, DCM/MeOH, 98:2 to 95:5), which furnished 207 mg (yield 74%) of the title compound as a red viscous oil. **¹H NMR (400 MHz, Chloroform-*d*)** δ 8.38 (t, *J* = 13.4 Hz, 1H), 7.62 – 7.51 (m, 2H), 7.42 (t, *J* = 7.5 Hz, 2H), 7.32 (d, *J* = 7.2 Hz, 2H), 7.22 (t, *J* = 7.5 Hz, 2H), 6.59 (dd, *J* = 13.5, 2.5 Hz, 2H), 4.14 – 4.03 (m, 4H), 3.67 – 3.55 (m, 60H), 3.54 – 3.46 (m, 8H), 3.44 – 3.38 (m, 2H), 3.35 (s, 6H), 3.32 (t, *J* = 6.7 Hz, 2H), 2.78 – 2.69 (m, 4H), 2.12 – 2.01 (m, 4H), 1.96 – 1.80 (m, 4H), 1.70 (s, 12H). **HRMS ESI (*m/z*):** [M+Na]²⁺/2 calcd. for 710.4217; found 710.4237. **HRMS ESI (*m/z*):** [M+2Na]³⁺/3 calcd. for 481.2775; found 481.2780.

“Clickable” cyanine 5 cross-linker Cy5L. The compound **Cy5L** was synthesized by the same protocol



as described above for the compound **Cy3L**. The product was purified by gradient column chromatography (SiO₂, DCM/MeOH, 98:2 to 95:5), which yielded 229 mg (65%) of the title compound as blue viscous oil. **¹H NMR (400 MHz, Chloroform-*d*)** δ 7.82 (t, *J* = 13.0 Hz, 2H), 7.40 – 7.33 (m, 4H), 7.31 (d, *J* = 7.4 Hz, 2H), 7.22 – 7.15

(m, 2H), 6.90 – 6.74 (m, 1H), 6.29 (d, *J* = 13.5 Hz, 2H), 4.09 – 4.00 (m, 4H), 3.66 – 3.57 (m, 60H), 3.55 – 3.50 (m, 8H), 3.40 – 3.36 (m, 2H), 3.35 (s, 6H), 3.33 – 3.29 (m, 2H), 2.69 – 2.58 (m, 4H), 2.11 – 2.01 (m, 4H), 1.89 – 1.80 (m, 4H), 1.66 (s, 12H). **HRMS ESI (*m/z*):** [M]⁺ calcd. for C₇₃H₁₁₉N₁₀O₁₈, 1423.8698; found, 1423.8705.

Preparation of fluorescent NPs

The cyanine (Cy3L, either Cy5L) derivative was dissolved at 10mM in Milli-Q® water. The concentration was measured by photometry using the extinction coefficient for Cy3 and Cy5 derivatives 150000 and 250000 M⁻¹ cm⁻¹ in methanol, respectively. Calixarene derivative was dissolved at 10 mM in Milli-Q® water. Copper sulfate and sodium ascorbate were dissolved in water at 100mM.

The **CX8TP** derivative (15μL of 10 mM, 150μM final) was injected quickly to a solution of sodium sulfate (919μL, 75mM final) in 1.5mL eppendorf tube under stirring (shaking) using a micropipette, then the solution was vortexed for 30 s. To the obtained solution of micellar NPs copper sulfate solution (12 μL of 100mM, 1.2 mM final) and sodium ascorbate (24 μL of 100mM, 2.4mM final) were added. After vortexing for another 30 s, the formation of Cu(I) precipitate (stable colloid of microparticles) was observed. Finally, the solution of fluorescent bi-functional cross-linker (**Cy3L**, either **Cy5L**) was added *via* quick injection using a micropipette (30 μL of 10mM, 300 μM final) and the obtained mixture was vortexed for another 30 s. The reaction mixture was stirred under gentle shaking (Thermomixer comfort, Eppendorf, 900 rpm) at 30 °C for 24 h. After completion, when precipitate of Cu(I) microparticles disappeared and the solution became clear, the reaction mixture was

diluted two times using 75mM solution of sodium sulfate and dialyzed against 1000x volume excess of Milli-Q® water (2000mL) using dialysis tubing cellulose membrane (D9652 Sigma, typical molecular weight cut-off = 14,000 Da). All dialysis systems were covered by aluminum foil to protect fluorescent micellar NPs against sunlight. For the DLS and AFM measurements of cross-linked particles, to remove possible aggregates the obtained solution of prepared nanoparticles was filtered through a 0.1 µm PVDF NS “Ultrafree®-CL” centrifugal filter unit (Merck Millipore). In the case of FRET NPs, the same synthetic protocol was followed, but in this case Cy3L and Cy5L were taken in different ratios, 10/1, 25/1 and 50/1, while keeping the total concentration of cross-linker 300 µM. Then the samples were dialyzed as described above.

To determine the yield of cross-linking reaction we have measured the absorption spectra. The yield of the cross-linking “click” reaction was determined as follows:

$$\varphi = \frac{A_{\text{after dialysis}} - A_{\text{after dialysis}}^{\text{control}}}{A_{\text{before dialysis}}} \quad (\text{Eq. 1})$$

Where $A_{\text{after dialysis}}$ is peak absorbance of the dye in the cross-linked sample after dialysis, $A_{\text{after dialysis}}^{\text{control}}$ is the peak absorbance of the dye in the sample without Cu-catalysis after dialysis and $A_{\text{before dialysis}}$ is peak absorbance of the dye in the cross-linked sample before dialysis.

Optical spectroscopy

Absorption and emission spectra were recorded on a Cary 400 Scan UV-visible spectrophotometer (Varian) and a FluoroMax-4 spectrofluorometer (Horiba JobinYvon) equipped with a thermostated cell compartment, respectively. For standard recording of fluorescence spectra, the excitation wavelength was set to 520 and 605 nm for Cy3L and Cy5L dyes, respectively. The fluorescence spectra were corrected for detector response and lamp fluctuations. Fluorescence quantum yields were calculated using rhodamine B in water (QY = 31%)^[1] with an absorbance of 0.1 at 520 nm as a reference. The quantum yield calculations included correction for the solvent refractive index. The refractive indexes of glycerol-methanol mixtures at 298.15 K were taken elsewhere.^[2] Viscosities of glycerol at different temperatures were taken elsewhere.^[3] Hydrodynamic diameter and zeta-potential measurements were performed on a Zetasizer Nano ZS (Malvern Instruments S.A.) with a laser source at 633 nm.

Time-resolved fluorescence spectroscopy

Time-resolved fluorescence measurements were performed with the time-correlated, single-photon counting technique using the frequency doubled output of a Ti-Sapphire laser (Tsunami, Spectra Physics), pumped by a Millennia X laser (Tsunami, Spectra Physics). The excitation wavelength was set at 320 nm. The fluorescence decays were collected at the magic angle (54.7°) of the emission polarizer. The single-photon events were detected with a microchannel plate Hamamatsu R3809U photomultiplier coupled to a Philips 6954 pulse preamplifier and recorded on a multichannel analyzer (Ortec 7100) calibrated at 25.5 ps/channel. The

instrumental response function was recorded with a polished aluminium reflector, and its full-width at half-maximum was 50 ps. Time-resolved decays were analyzed both by the iterative reconvolution method and the Maximum Entropy Method (MEM). The goodness of the fit was evaluated from the χ^2 values, the plots of the residuals and the autocorrelation function.

Fluorescence correlation spectroscopy (FCS) and data analysis

FCS measurements were performed on a two-photon platform including an Olympus IX70 inverted microscope, as described previously.^[4] Two-photon excitation at 760 nm (3 mW laser output power) was provided by an InSight DeepSee laser (Spectra Physics). The measurements were carried out in a 96-well plate with a glass bottom, using a 200- μ L volume per well. The focal spot was set about 20 μ m above the coverslip. The normalized autocorrelation function, $G(\tau)$ was calculated online by an ALV-5000E correlator (ALV, Germany) from the fluorescence fluctuations, $\delta F(t)$, by $G(\tau) = \langle \delta F(t) \delta F(t + \tau) \rangle / \langle F(t) \rangle^2$ where $\langle F(t) \rangle$ is the mean fluorescence signal, and τ is the lag time. Assuming that lipid nano-droplets diffuse freely in a Gaussian excitation volume, the correlation function, $G(\tau)$, calculated from the fluorescence fluctuations was fitted according to Thompson:^[5]

$$G(\tau) = \frac{1}{N} \left(1 + \frac{\tau}{\tau_d} \right)^{-1} \left(1 + \frac{1}{S^2} \frac{\tau}{\tau_d} \right)^{-1/2}$$

where τ_d is the diffusion time, N is the mean number of fluorescent species within the two-photon excitation volume, and S is the ratio between the axial and lateral radii of the excitation volume. The excitation volume is about 0.34 fL and S is about 3 to 4. Typical data recording times were 5 min, using freshly prepared PLGA NPs without further dilution. The measurements were done with respect to a reference 6-carboxytetramethylrhodamine (TMR from Sigma-Aldrich) in water. The hydrodynamic diameter, d , of NPs was calculated as: $d_{\text{NPs}} = \tau_d(\text{NPs}) / \tau_d(\text{TMR}) \times d_{\text{TMR}}$, where d_{TMR} is a hydrodynamic diameter of TMR (1.0 nm). Concentration of NPs was calculated from the number of species by: $C_{\text{NPs}} = N_{\text{NPs}} / N_{\text{TMR}} \times C_{\text{TMR}}$, using a TMR concentration of 50 nM.

AFM measurements

AFM measurements were performed using a Solver-Pro-M (NT-MDT) instrument. The measurements were done in liquid phase in tapping mode. Cantilevers were CSG01 (NT-MDT) with a tip curvature radius of 10 nm, the typical resonance frequency was 23kHz. The NPs were prepared as described before. 100 μ L of diluted suspension of micellar NPs was deposited on the freshly cleaved mica surface. After 30 min, the solution was removed using a filter paper and then replaced with 100 μ L Milli-Q water. The obtained sample was imaged in liquid phase, using the tapping mode with a scan rate of 1Hz and a resolution of 512x512.

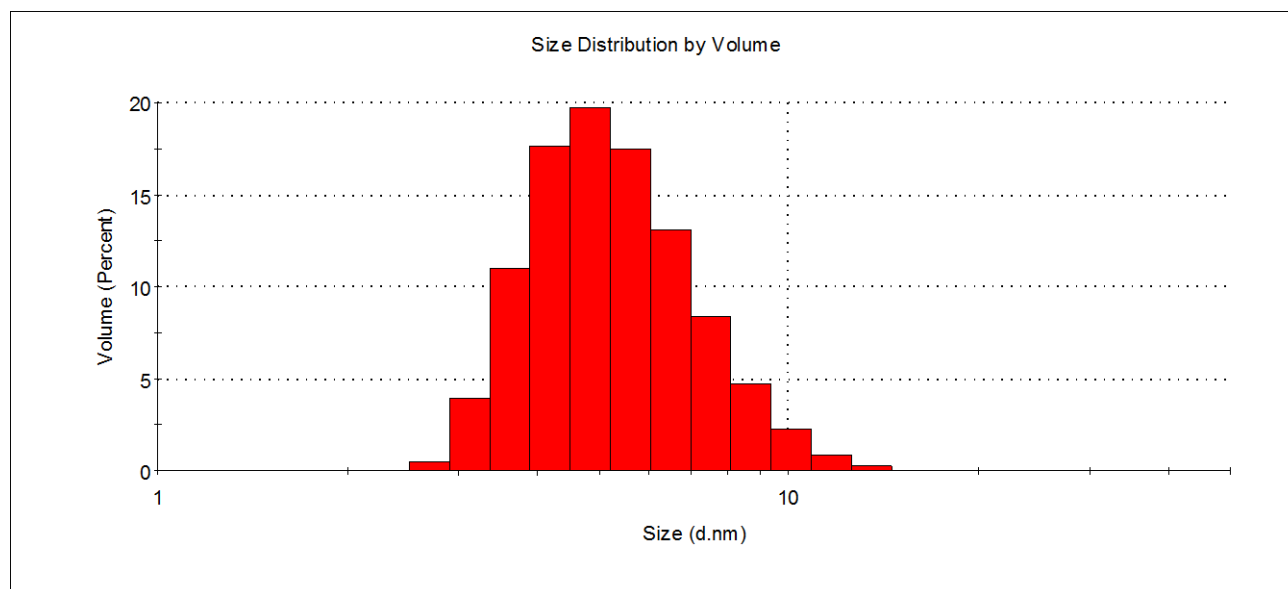
Single-particle imaging

The 300 μL of CX8/Cy3L 1:2 crosslinked micelles after 250,000X dilution were deposited on a glass surface of Lab-Tek® plate. After 30 min of incubation, the NPs solution was removed and the glass surface was washed once with Mili-Q® water (Millipore). To check fluorogenic behavior of micellar NPs in viscous media the 300 μL of glycerol were added into the new well of Lab-Tek® plate and, after that, 6 μL of CX8/Cy3L 1:2 crosslinked micelles were injected into glycerol to achieve 10,000X dilution from the initial NPs concentration obtained after dialysis. The obtained solution of NPs in glycerol was incubated for 30 min at room temperature before imaging. Quantum dots (QDot-585 streptavidine conjugate, Life Technologies) were deposited on the glass covered with polyethyleneimine (PEI) as described before.^[6] Single particle measurements were performed in the TIRF (Total Internal Reflection Fluorescence) mode on a home-made wide-field setup based on an Olympus IX-71 microscope with an oil immersion objective (NA = 1.49, 100X). A DPPS (Cobolt) continuous wave (CW) laser emitting at 532 nm was used for excitation. The laser intensity set to 5 W cm⁻² using a polarizer and a half-wave plate (532 nm). The fluorescence signal was recorded with an EMCCD (ImagEM Hamamatsu). The presented images were an average of the first 30 frames recorded with an acquisition time of 30.67 ms per frame.

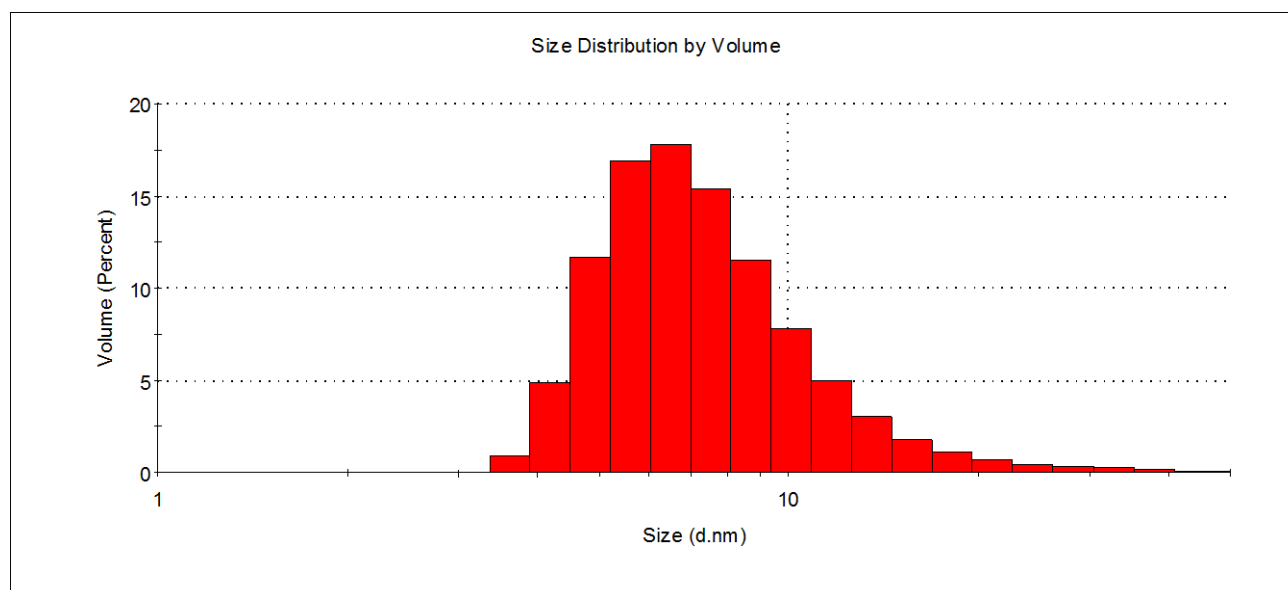
Cellular studies

HeLa cells (ATCC® CCL-2) were grown in Dulbecco's modified Eagle medium (DMEM, Gibco-Invitrogen), supplemented with 10% fetal bovine serum (FBS, Lonza) and 1% antibiotic solution (penicillin-streptomycin, Gibco-Invitrogen) at 37° C in humidified atmosphere containing 5% CO₂. Cells were seeded onto a chambered coverglass (IBiDi) at a density of 5×10⁴ cells/well 24h before the microscopy measurement. For imaging, the culture medium was removed and the attached cells were washed with Opti-MEM (Gibco-Invitrogen). Then, a freshly prepared solution of micellar NPs (at 20-fold dilution of the original formulation, corresponding to 0.5 μM dye) in Opti-MEM was added to the cells and incubated for different time periods. Cell membrane staining with wheat germ agglutinin-Alexa488 was done for 10 min at rt before the measurements. Fluorescence images were taken on a Leica TSC SPE confocal microscope. The microscope settings for Cy3L cross-linked micelles were: 561 nm laser source with 567-700 nm detection range for imaging micellar NPs and 488 nm excitation with 503-550 nm emission range for imaging plasma membrane marker WGA-AlexaFluor®488 or (green) or Mito-Tracker® green. In case of FRET micelles, signal from the FRET donor was recorded at 576-640 nm (Cy3 channel), while the signal from the FRET acceptor was recorded at 650-750 nm (Cy5 channel). Excitation wavelength for the FRET micelles was 561 nm. Images of acceptor (Cy5L) were acquired using excitation at 635 nm (laser source) with detection of emission at 650-750 nm.

Additional data



(a)



(b)

Figure S1. Distribution of the hydrodynamic diameters of (a) CX8TP micelles in 75mM sodium sulfate and (b) CX8TP/Cy3L 1:2 crosslinked micelles in 75mM sodium sulfate.

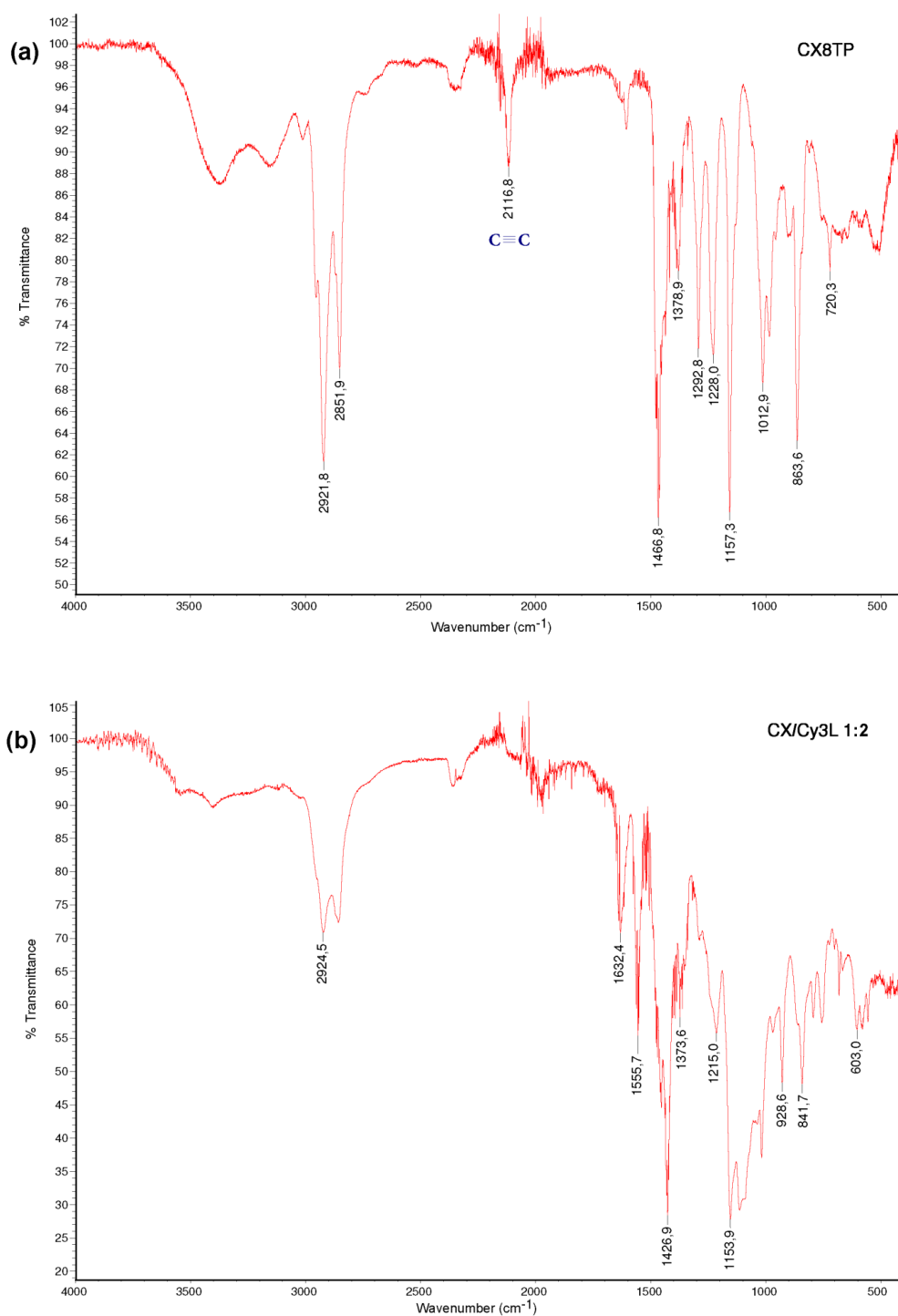


Figure S2. FT-IR spectra of (a) CX8TP and (b) CX8TP/Cy3L 1:2. IR spectra were recorded on a Nicolet 380 FT-IR spectrometer from Thermo Electron Corporation as a dried solid from aqueous solution on a diamond plate.

Atomic force microscopy data

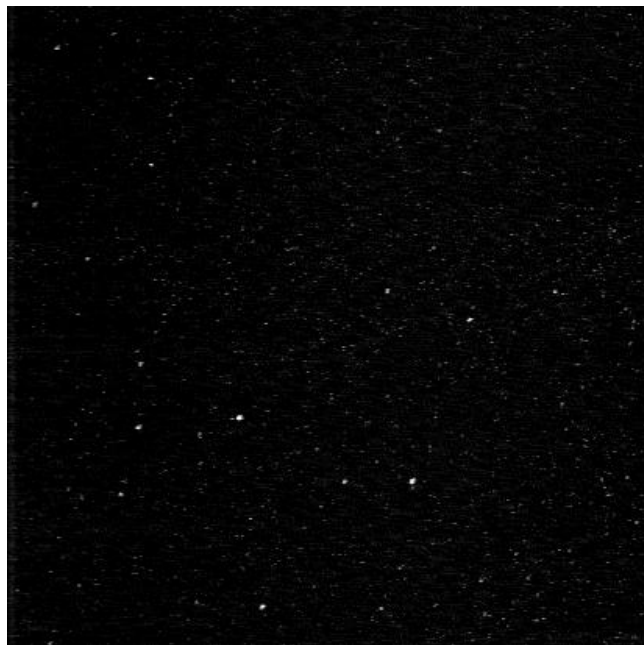


Figure S3. AFM image of shell-cross-linked micelles build from Calix with Cy5L at 1:2 molar ratio deposited on mica.

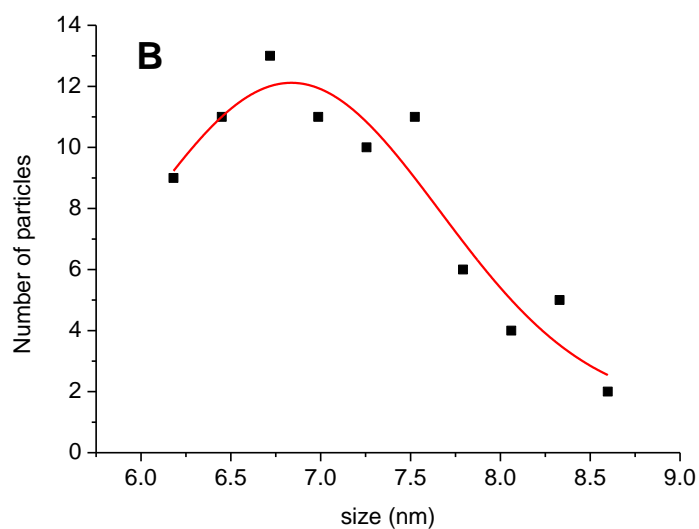
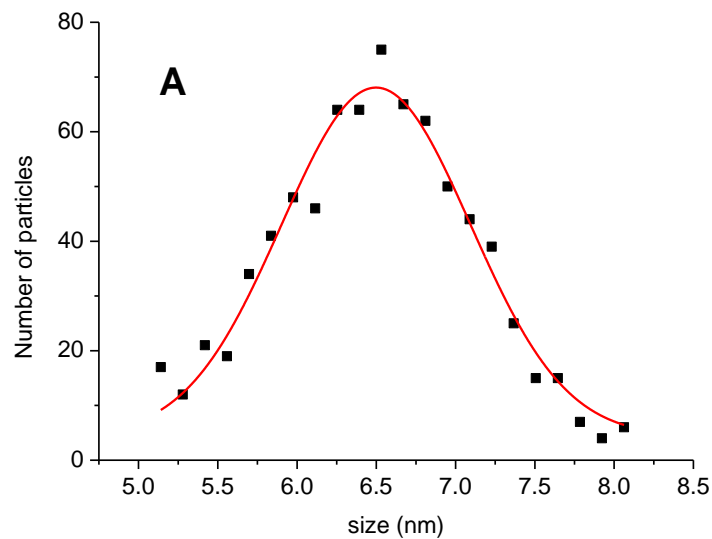


Figure S4. AFM size statistics of micellar NPs after cross-linking with dyes: (A) Cy3L / CX8TP 2/1, average size 6.5 nm; (B) Cy5L / CX8TP 2/1, average size 7 nm. Particle diameter was estimated from the height measurements. Gaussian model was applied for the function fitting of the size distribution.

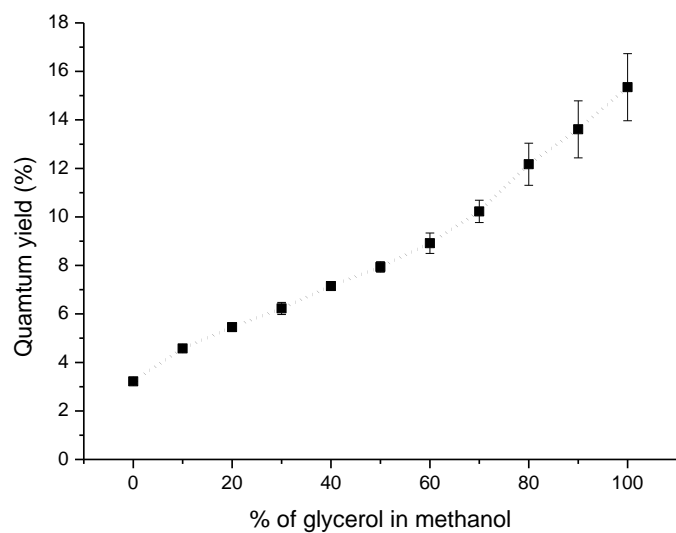


Figure S5. Dependence of fluorescence quantum yield of Cy3L / CX8TP cross-linked micelles on the content of glycerol in glycerol-methanol mixture.

Absorption and excitation spectral data

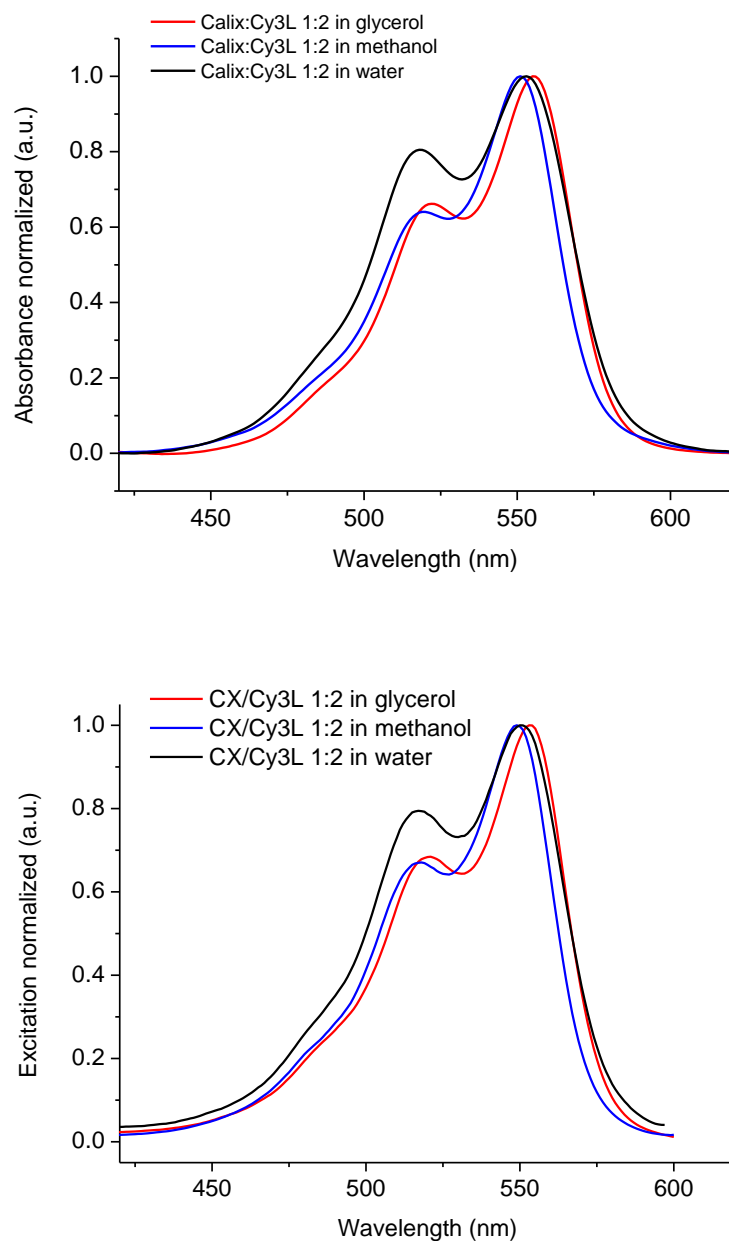


Figure S6. Normalized absorption (top) and excitation (bottom) spectra of Cy3L / CX8TP cross-linked micelles in different media. Peak absorbance of the samples was 0.06-0.08. Emission wavelength was 610 nm.

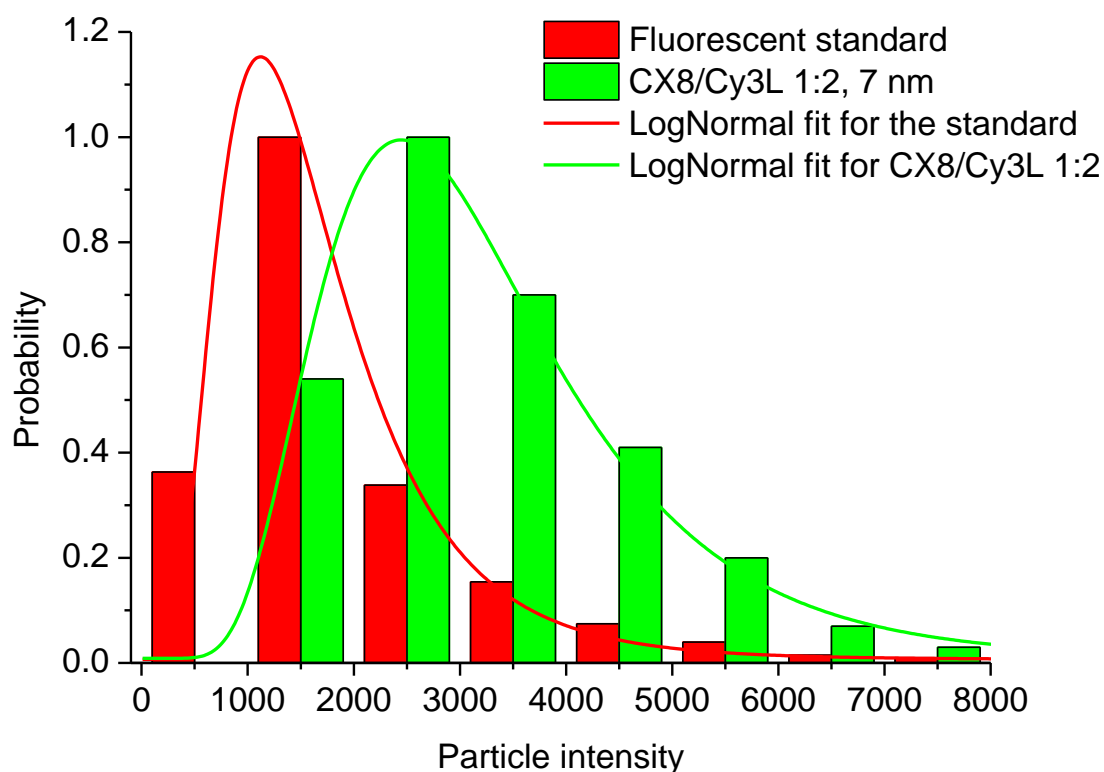


Figure S7. Analysis of the intensity distribution of cross-linked (polymerized) micelles (CX8TP/Cy3L 1:2, 7 nm) versus fluorescent standard (Qdot® 585 Nanocrystals, diameter ~15–20 nm).¹ The polymerized micelles were imaged in glycerol on glass surface using single-molecule setup with 532-nm laser as an excitation source. Qdot® 585 Nanocrystals were imaged on glass surface covered by PEI at 100pM concentration using the same experimental settings.

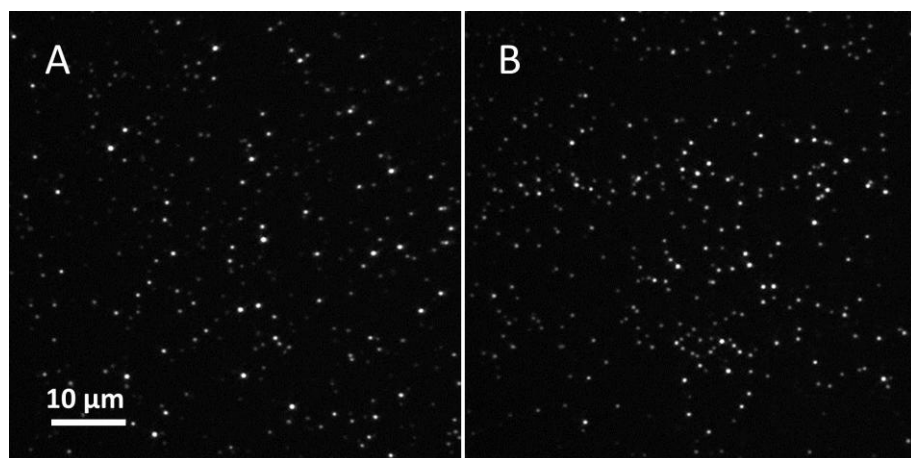


Figure S8. Single particle properties of fluorescent micelles. Wide-field fluorescence images of the shell-cross-linked micelles (CX8TP and Cy3L at 1:2 molar ratio) deposited on glass in water (A) and QD585 deposited on PEI/glass surface (B). The laser power density was 10 W cm⁻² at 532 nm.

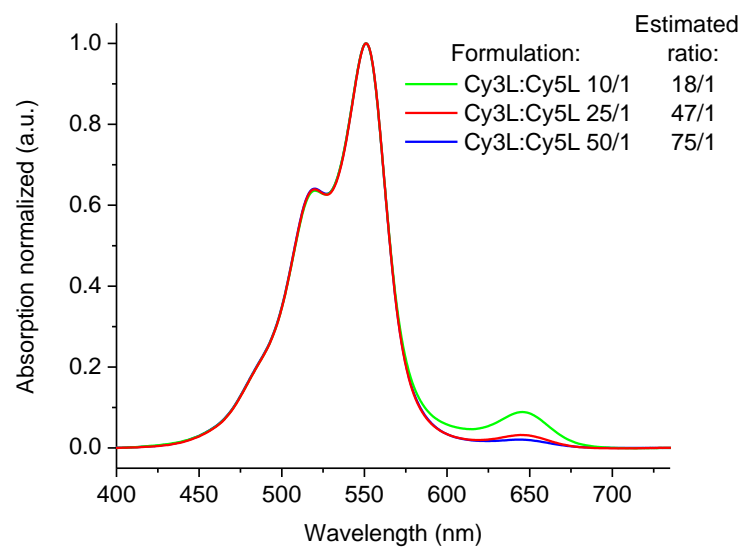


Figure S9. Absorption spectra of cross-linked FRET micelles at different Cy3L/Cy5L ratios. Numbers on the right present the Cy3L/Cy5L ratios estimated based on the absorption spectra.

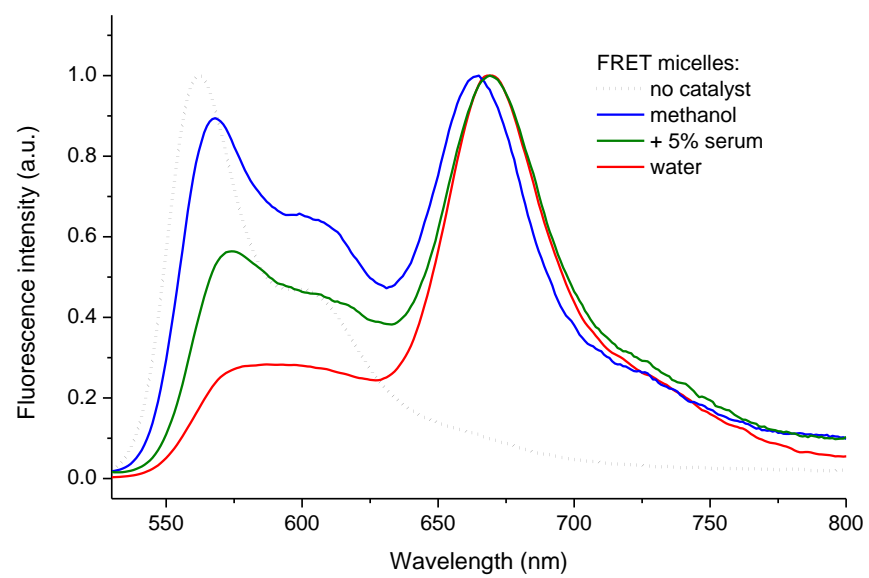


Figure S10. FRET micelles and a control without cross-linking (no catalyst) in different media. Cy3L/Cy5L ratio was 25/1.

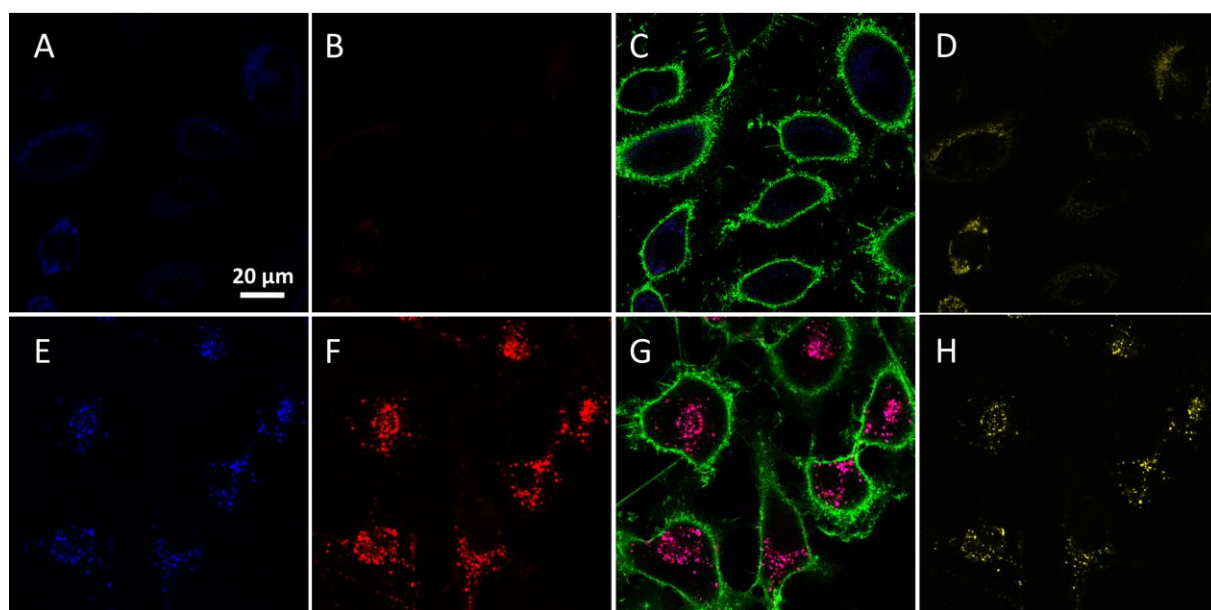


Figure S11. Fluorescence confocal imaging of cells incubated for 3h at 37°C with non-cross-linked (A-D) and cross-linked (E-H) FRET micelles. (A, E) Signal from the FRET donor recorded at 576-640 nm (Cy3 channel). (B,F) Signal from the FRET acceptor recorded at 650-750 nm (Cy5 channel). (C,G) Merged images of donor and FRET channels, where green corresponds to plasma membrane staining with WGA-Alexa Fluor488 (50nM). Excitation wavelength for the micelles and WGA-Alexa Fluor488 was 561 and 488 nm, respectively. (D,H) Images of acceptor (Cy5L): excitation at 635 nm (laser source) with detection at 650-750 nm. Concentration of Cy3L in free form and in cross-linked micelles was 0.5 μ M. Cy3L/Cy5L ratio was 10/1.

Table S1. Fluorescence lifetime data of CX/Cy3L NPs in different solvents.^a

Solvent	τ_{mean} (ns)	τ_1 (ns)	α_1 %	τ_2 (ns)	α_2 %	τ_3 (ns)	α_3 %
glycerol	1.265	0.287	41	2.021	59	-	-
MeOH	0.155	0.143	78	0.411	21	1.097	1
water	0.149	0.112	71	0.406	22	0.91	7

^a τ_{mean} is a mean fluorescence lifetime; τ_1 , τ_2 and τ_3 (ns) are different decay times, α_1 , α_2 and α_3 are the relative amplitudes, obtained after analysis of fluorescence decay data by method of maximum entropy; the error for all values is $\pm 10\%$ due to the precision of the measurements.

Fluorescence correlation spectroscopy measurements of cross-linked micelles

Based on the correlation time of the CX/Cy3L micelles (0.111 ms) in water, we could estimate that their hydrodynamic diameter is 5.9 nm, in perfect agreement with DLS, AFM and TEM data. Secondly, the brightness of these micelles in water is close to 0.76 molecule of tetramethyl-rhodamine (TMR). Taking into account the two-photon absorption cross-section (σ_2) of rhodamine at 760 nm (85 GM) and its quantum yields in water (0.4) and those for Cy3L dye (25 GM and 0.02, respectively),^[7] we calculated that 0.76 rhodamine is equivalent of 52 Cy3L dyes in aggregated state. This calculation suggests that the micelle contains around 52 dyes on its surface. This value is on the same order of magnitude as we take into account that the aggregation number 40 of a parent calixarene amphiphile CX8,^[21] the expected number of Cy3L molecules grafted at the micellar surface is 80, and crosslinking degree for Cy3L (59%), we could finally obtain the value of 47 Cy3L dyes on micelle surface, which is in good agreement with the one obtained from the FCS measurements.

Table S2. Two-photon fluorescence correlation spectroscopy (FCS) data of NPs in comparison to tetramethyl-rhodamine dye (TMR).^a

	TMR	CX/Cy3L
τ_{corr} , ms	0.019	0.111
N	12	33
Bri, kHz	0.43	0.33
size, nm	1	5.9
Bri/TMR	1	0.76
Bri/Cy3	68	52
Conc. nM	50	135
dye/NP	1	ND

^a τ_{corr} – correlation time, N – number of emissive species per excitation volume, Bri – brightness per particle, Conc. – concentration of species.

NMR and mass spectra

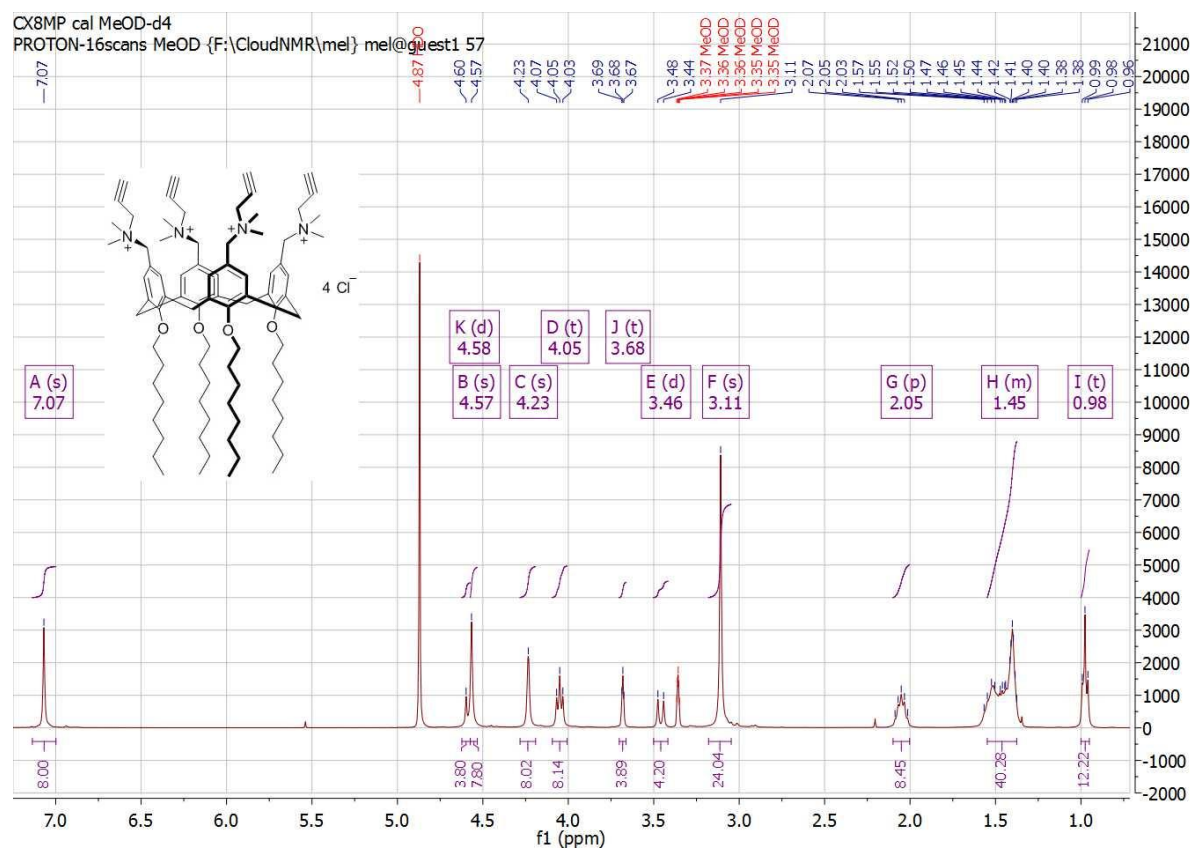


Figure S12. ¹H NMR spectrum of compound CX8TP.

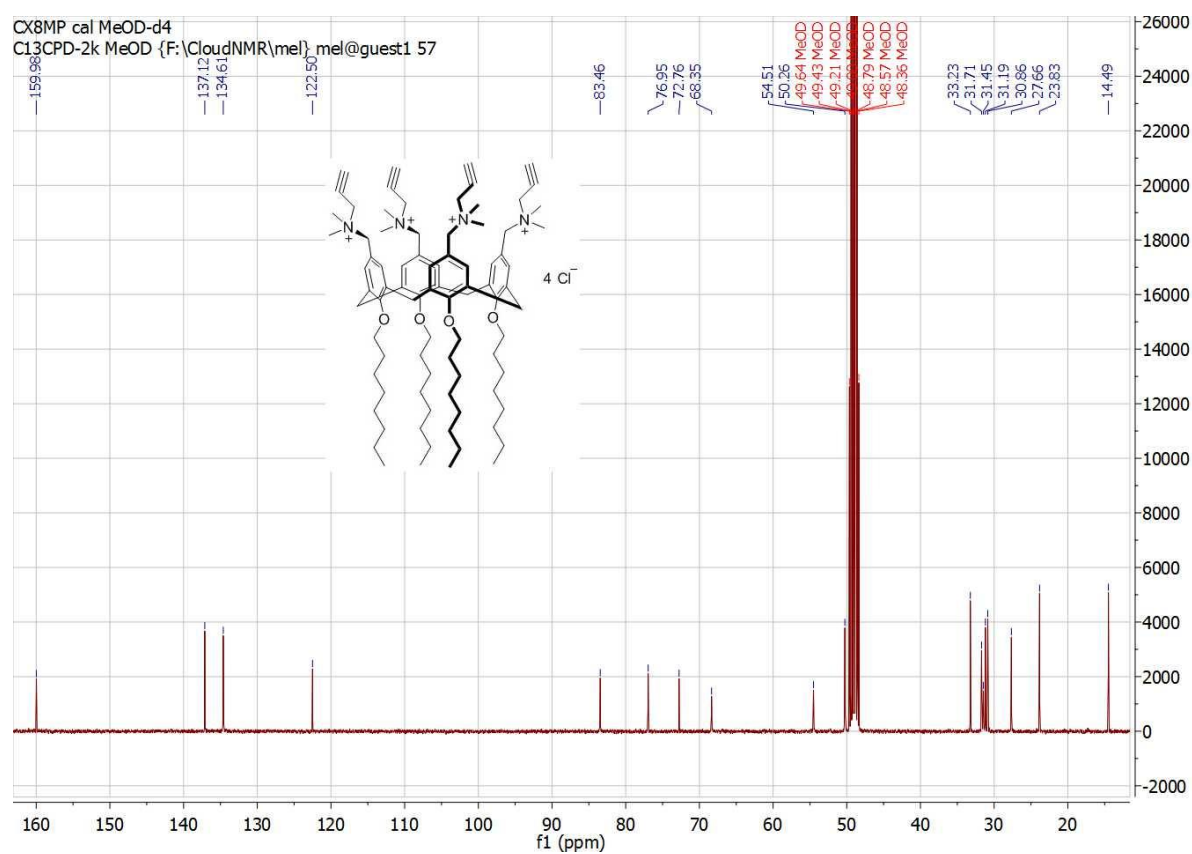
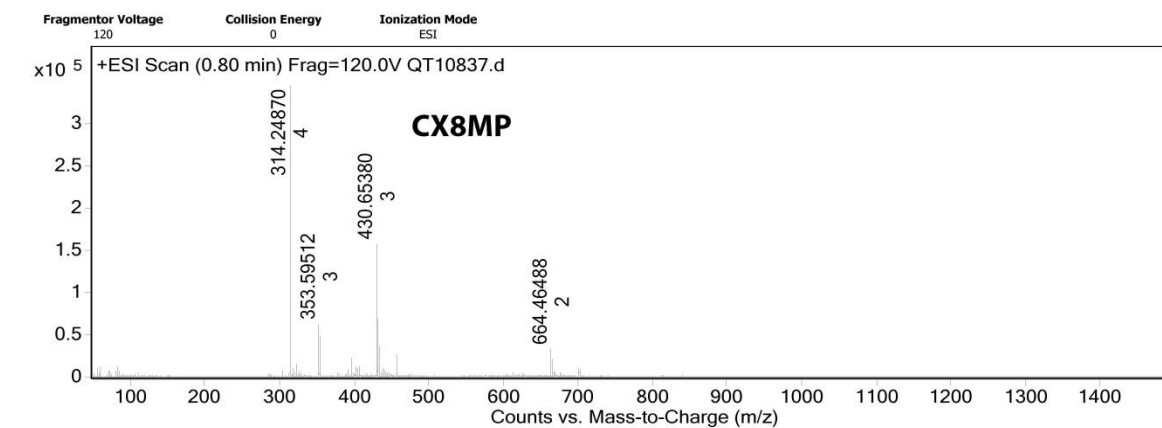


Figure S13. ^{13}C NMR spectrum of compound **CX8TP**.

Qualitative Analysis Report



Peak List				
m/z	z	Abund	Formula	Ion
314.2487	4	348057.6	C84 H128 N4 O4	M+4
314.49935	4	326992.8	C84 H128 N4 O4	M+4
314.75022	4	149951.3	C84 H128 N4 O4	M+4
315.00067	4	49258.4	C84 H128 N4 O4	M+4
353.59512	3	62831.4		
353.92976	3	48291.9		
430.6538	3	161878.6		M*+3
430.98895	3	155569		M*+3
431.32153	3	119962.1		M*+3
431.65533	3	69351		M*+3

Figure S14.HRMS data of compound **CX8TP**.

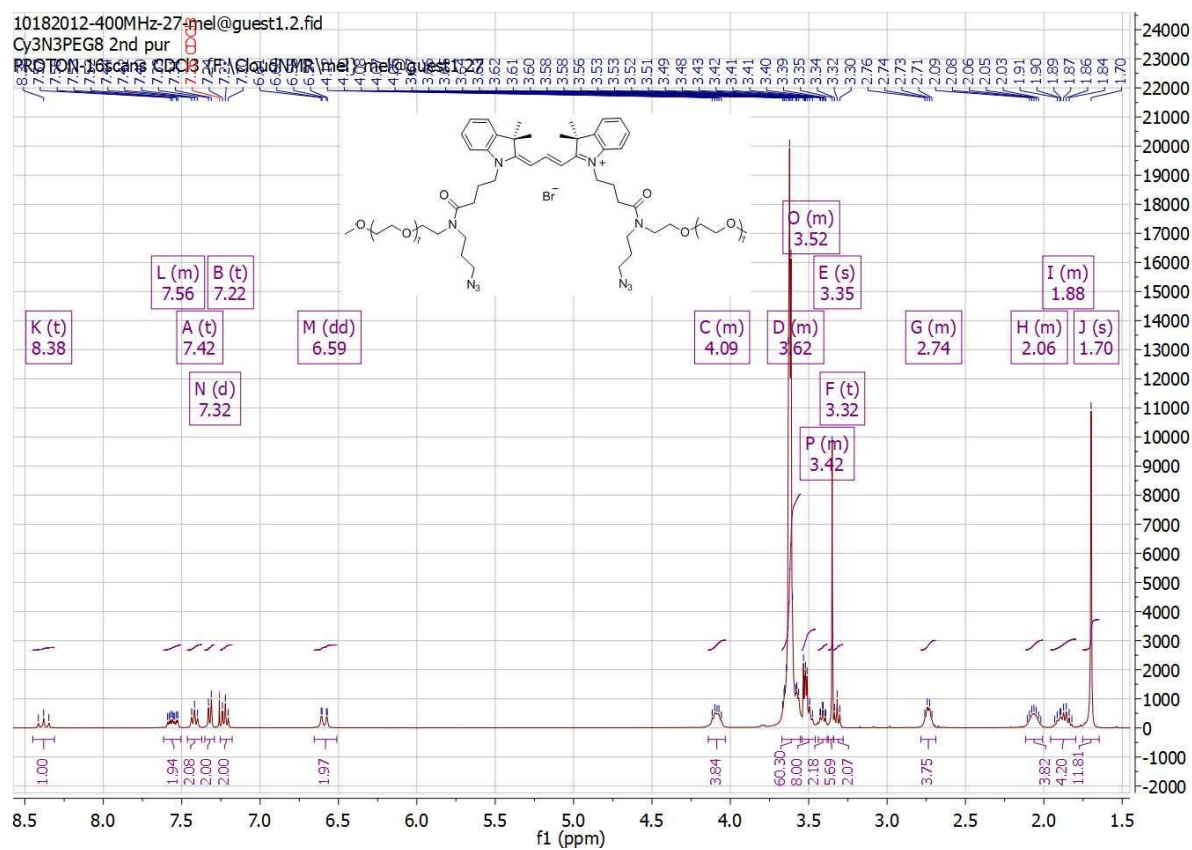


Figure S17. ^1H NMR spectrum of compound **Cy3L**.

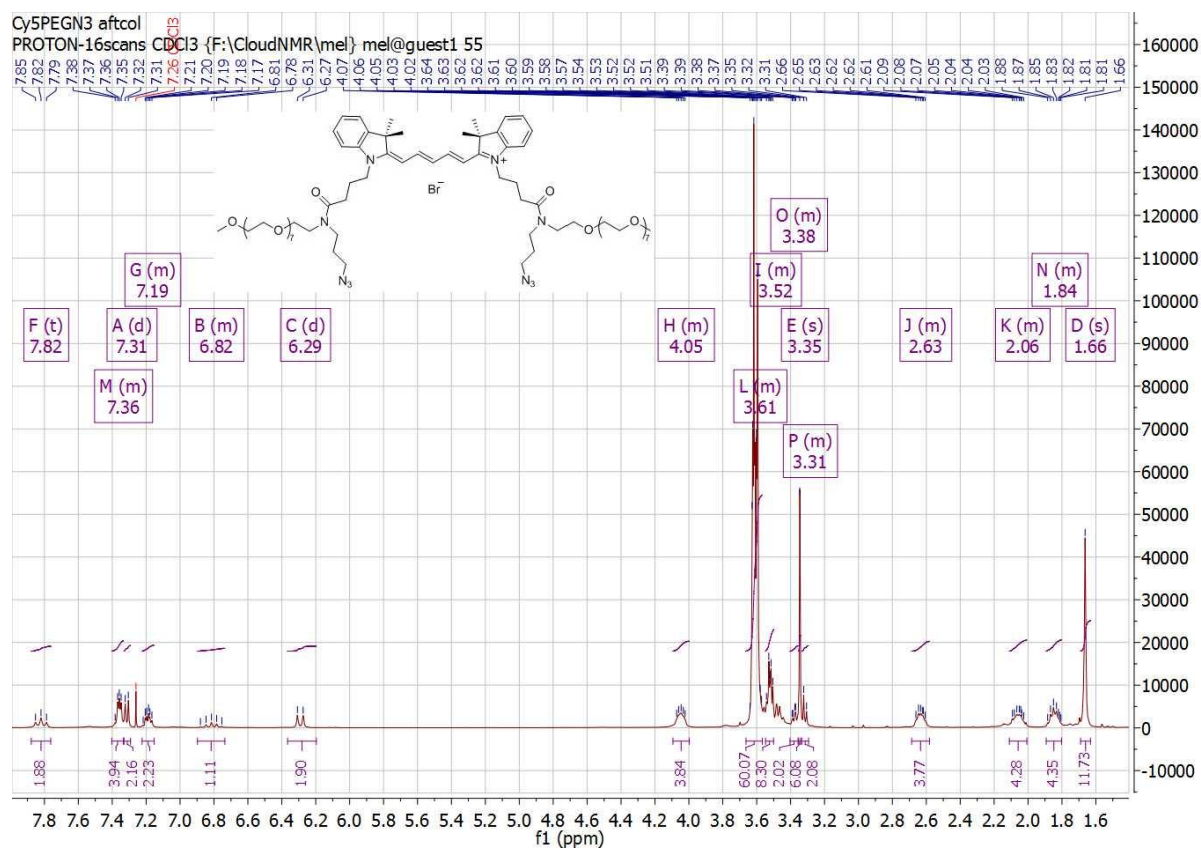


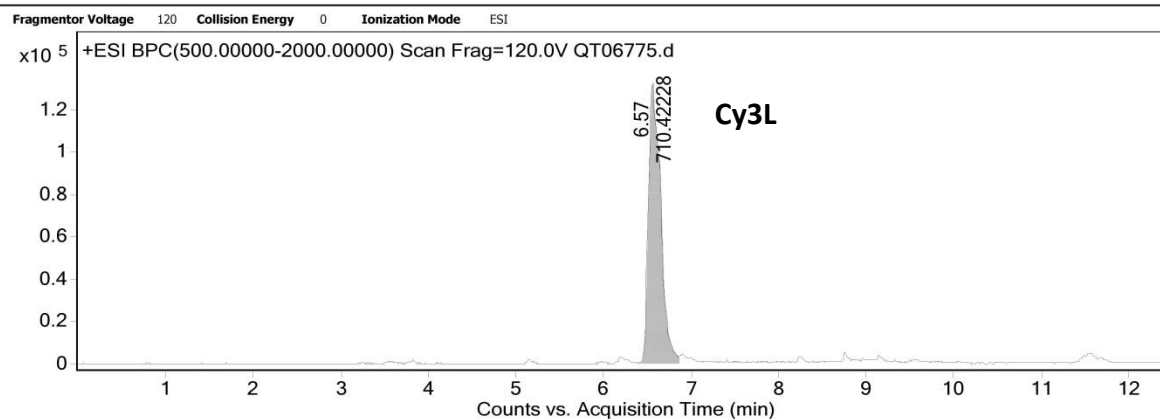
Figure S18. ¹H NMR spectrum of compound Cy5L.

Qualitative Analysis Report

Data Filename	QT06775.d	Sample Name	u0160
Inj. Vol.	0.3	Position	P2-B1
Instrument Name	SCA IIIkirch QToF	User Name	
Acq Method	C18-2, 1x5x1,8.m	Acquired Time	6/12/2013 10:01:59 AM
IRM Calibration Status	Success	DA Method	C18-2, 1x5x1,8.m
Comment			

Sample Group Info.

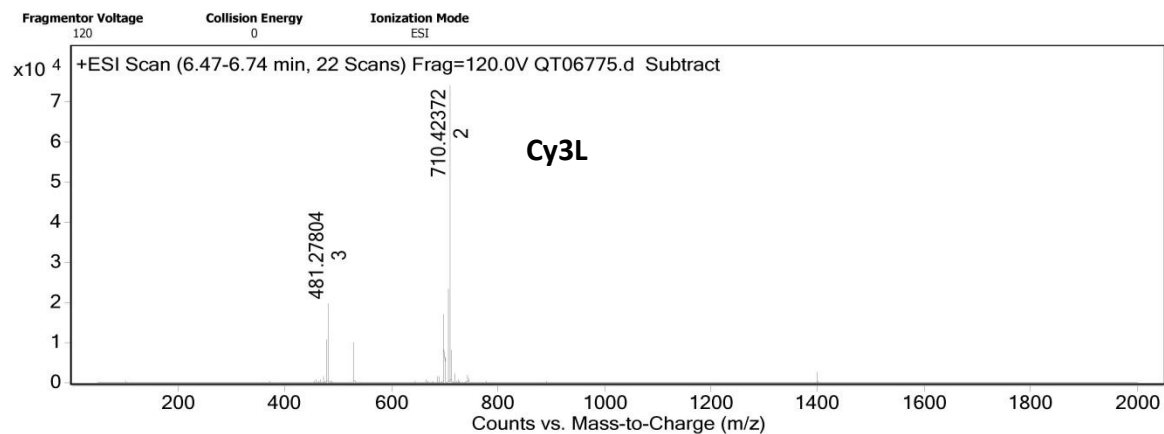
User Chromatograms



Integration Peak List

Start	RT	End	Height	Area	Area %	AreaSum%
6.4	6.57	6.86	132424	1310491	100	100

Qualitative Analysis Report



Peak List

m/z	z	Abund	Formula	Ion
479.62569	3	10805.9		
481.27804	3	19898.8		
481.61204	3	16398.9		
699.43214	2	17333.5	C71 H118 N10 O18	(M+2H)+2
699.93373	2	14699.6	C71 H118 N10 O18	(M+2H)+2
707.94515	2	23429.4	C71 H121 N11 O18	(M+H+NH4)+2
708.44674	2	19562.3	C71 H121 N11 O18	(M+H+NH4)+2
710.42372	2	74653.6	C71 H117 N10 O18	(M+H+Na)+2
710.92493	2	61534.4	C71 H117 N10 Na O18	(M+H+Na)+2
711.42602	2	25391	C71 H117 N10 Na O18	(M+H+Na)+2

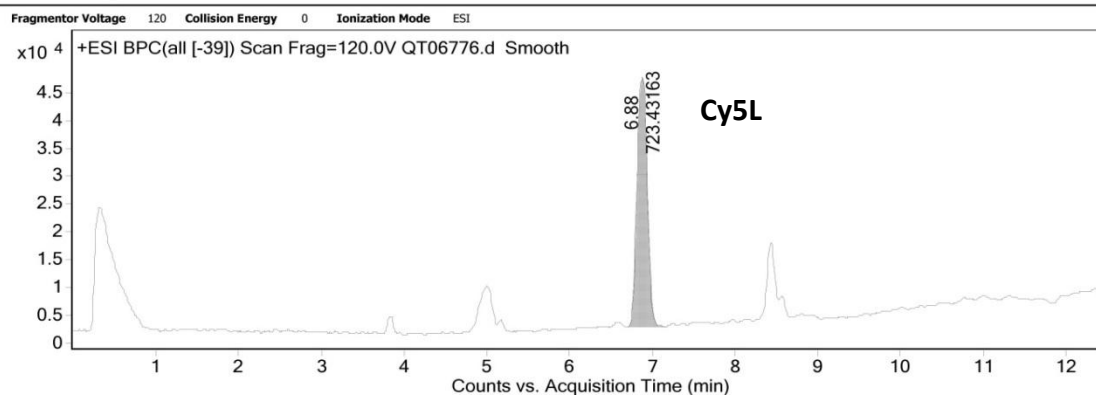
Figure S19. HRMS data of compound **Cy3L**.

Qualitative Analysis Report

Data Filename	QT06776.d	Sample Name	u0209
Inj. Vol.	0.5	Position	P2-B2
Instrument Name	SCA IIIkirch QToF	User Name	
Acq Method	C18-2,1x5x1,8.m	Acquired Time	6/12/2013 10:18:54 AM
IRM Calibration Status	Success	DA Method	C18-2,1x5x1,8.m
Comment			

Sample Group Info.

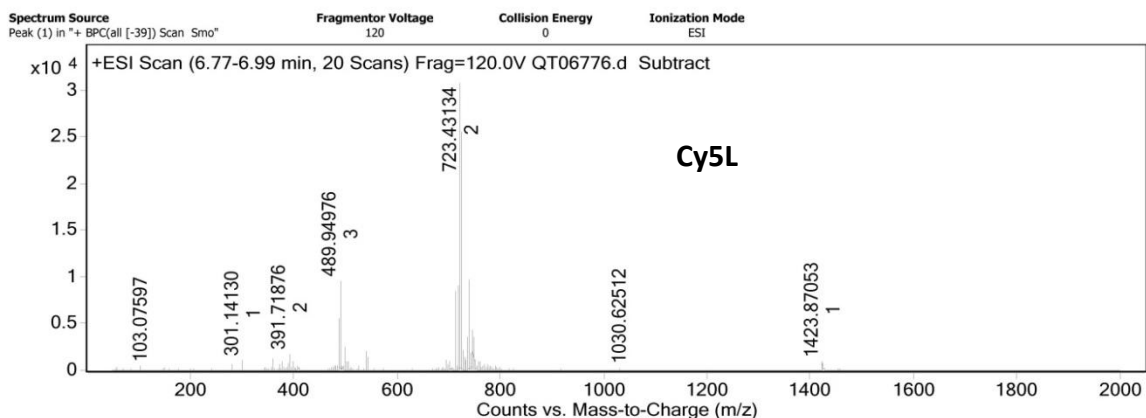
User Chromatograms



Integration Peak List

Start	RT	End	Height	Area	Area %	AreaSum%
6.7	6.88	7.16	44637	406077	100	100

Qualitative Analysis Report



Peak List

m/z	z	Abund	Formula	Ion
489.94976	3	9662.5		
490.28464	3	8399.6		
712.43929	2	8403	C73 H120 N10 O18	(M+2H)+2
718.40089	2	9283.9		
720.9517	2	9547.5		
723.43134	2	30738	C73 H119 N10 Na O18	(M+H+Na)+2
723.93264	2	25555.9	C73 H119 N10 Na O18	(M+H+Na)+2
724.43384	2	11736.9	C73 H119 N10 Na O18	(M+H+Na)+2
739.42547		9904.3		
739.92773	2	8145.8		

Figure S20. HRMS data of compound **Cy5L**.

References

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- [7] C. Xu, W. W. Webb, *Journal of the Optical Society of America B-Optical Physics* **1996**, 13, 481-491.