Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information:

A novel biocompatible europium ligand for sensitive time-gated immunodetection

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Materials and methods:

Unless specified otherwise, all reactions were performed under an inert atmosphere of nitrogen with dry solvents under anhydrous conditions and monitored by TLC using aluminium plates coated with Merck Silica Gel 60 F_{254}, visualised using either UV light (254 or 366 nm) or a europium staining reagent [2 mM $EuCl_3$ solution]. Ratios of solvent systems for TLC and column chromatography are expressed in v/v as specified. General chemicals for organic synthesis were the highest grade available purchased from Sigma Aldrich Australia and they were used without further purification. ^1H and ^{13}C NMR spectra were recorded with Bruker Avance Spectrometer [400 MHz (^1H) and 100 MHz (^{13}C)] in $CDCl_3$ at 298 K. The solvent ^1H and ^{13}C signals, δ_H 7.26 for residual $CHCl_3$ and δ_C 77.0 for $CDCl_3$ were used as internal references. Flash chromatography was performed on silica gel (Merck silica gel 60, 40-63 μm). Reverse phase (RP) HPLC was performed using a Shimadzu apparatus consisting of a DGV-12A degasser, SIL-10AD auto injector, SPD-M10A tuneable absorbance detector. Analytical HPLC was performed using a Gemini-NY C18 column (5 μm, 4.6 mm ID, 250 mm) with a flow rate of 1.0 mL/min. Preparative HPLC was carried out using an Econosil C18 column (10 μm, 22 mm ID, 250 mm) with a flow rate of 9.0 mL/mi in water (0.05% TFA) and acetonitrile solvent system. UV-visible data was collected on a NanoDrop 2000 UV (Thermo Scientific) spectrometer. Luminescence data was captured on an Agilent Cary Eclipse Fluorescence Spectrophotometer. A pipette-size Sephadex column (G-50, 0.8-1.0 g, 7 cm length, 0.5 cm ID) was used for buffer exchange of antibody ($NaHCO_3$, 100 mM and pH 8.5) and purification of conjugated antibody (100 μg [1 μg/μL]. Antibody and conjugated antibody fractions were collected and analysed with a bio-photometer (absorption at 280 nm and 320 nm).

All bright-field, fluorescence and time-gated luminescence imaging was performed on an Olympus BX51 upright fluorescence microscope equipped with a UPLSAPO 100X OIL Objective Plan ApoChromat (Olympus part # N1480900). Conventional epifluorescence techniques using a FITC filter set cube were applied to capture the images shown in Figure 2 (A) and Figure 3 (A) of the manuscript. The camera was set for an exposure of 100 milliseconds at a sensitivity of ASA 200.

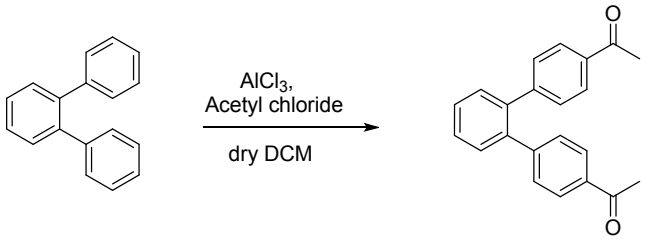
Time-gated luminescence was performed using the Gated Auto-synchronous Luminescence Detector (GALD), which was inserted into the DIC slot of the microscope.[1] TGL images were captured without a fluorescence filter using a DP-72 colour camera set for ASA speed of 200 and exposure period of 1.0 second; all images were stored as TIFF files as captured. FITC fluorescence imaging was carried out using a 100 W mercury arc lamp and a FITC filter set

with 100 millisecond exposure times. DAPI staining images were captured with the same source of UV lamp and DAPI filter with 10 ms exposure time. Printed microscope slides (G352104-W Teflon® printed slides, 21 wells, 4mm, white) supplied by ProSciTech Pty Ltd. *Giardia cysts* cells were purchased from BTF Pty Ltd (Sydney, Australia). *Giardia* monoclonal antibodies G2O3 and G2O3-conjugated to fluorescein isothiocyanate (G2O3-FITC) specific to the surface antigen of *Giardia cysts* were also purchased from BTF Pty Ltd. 4',6-Diamidino-2-phenylindole dihydrochloride hydrate (DAPI) were purchased from Sigma Aldrich Australia (D9642). Europium (III) chloride hexahydrate was purchased from Sigma Aldrich Australia (CAS:13759-92-7). Deuterium oxide (D_2O) was sourced from Sigma-Aldrich (151882) and water was deionized (Milli-Q). For details on the preparation of Fluorescence Enhancing Buffer (FEB) see page S34 of SI.

For purposes of comparison of photophysical properties we used BHHCT synthesized in our labs and commercial BHHCT purchased from Sigma Aldrich Australia (CAS:200862-70-0). *Synechococcus* cells (Cyanobacterium) were kindly provided by Ms. Deepa Varkey (Macquarie University).

Organic synthesis and characterization:

4,4'-Diacetyl-*o*-terphenyl 4



A solution of *o*-terphenyl (23 g, 100 mmol) in dry dichloromethane (20 mL) was added in a drop wise fashion, *via* use of a syringe, to a mixture of anhydrous aluminium chloride (30 g, 225 mmol) and acetyl chloride (20 g, 254 mmol) in dry dichloromethane (200 mL) at 0 °C over period of 1 h. The reaction mixture was stirred for 1 h at 0 °C, then warmed up to room temperature and stirring was continued overnight followed by 2 h reflux. After cooling, the reaction mixture was slowly added into a separating funnel containing ice-hydrochloric acid solution [ice (200 g) and hydrochloric acid (3 M, 100 mL)]. The aqueous layer was extracted with dichloromethane (2 x 100 mL) and the combined organic layers were washed with saturated brine (1 x 100 mL), dried over anhydrous magnesium sulfate, filtered and evaporated *in vacuo* to give crude product (30.0 g, > 97%). TLC analysis of crude product (hexane/ethyl acetate, 4/1) indicates the presence of the product 4,4'-diacetyl-*o*-terphenyl **4** (R_f = 0.3) as a major spot with minority of side product/s (R_f = 0.6) with no presence of starting material *o*-terphenyl (R_f = 0.85). The crude product was then dissolved in butanone by slightly heating (240 mL, about 8-10 mL per gram of crude product) then left at room temperature over night to give yellow crystals (23 g, 75%). Alternatively, silica gel column purification using CH_2Cl_2/hexane (1:1 to 4:1) was used to purify the crude product and after optimization 600 mg of crude product afforded 500 mg pure product. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.55 (s, 6H), 7.21 (d, *J* = 8.4 Hz, 4H), 7.41-7.49 (m, 4H), 7.80 (d, *J* = 8.4 Hz, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 26.5, 128.1, 128.2, 129.9, 130.5, 135.3, 139.4, 145.9, 197.6. The data are in agreement with those in the literature.[2]

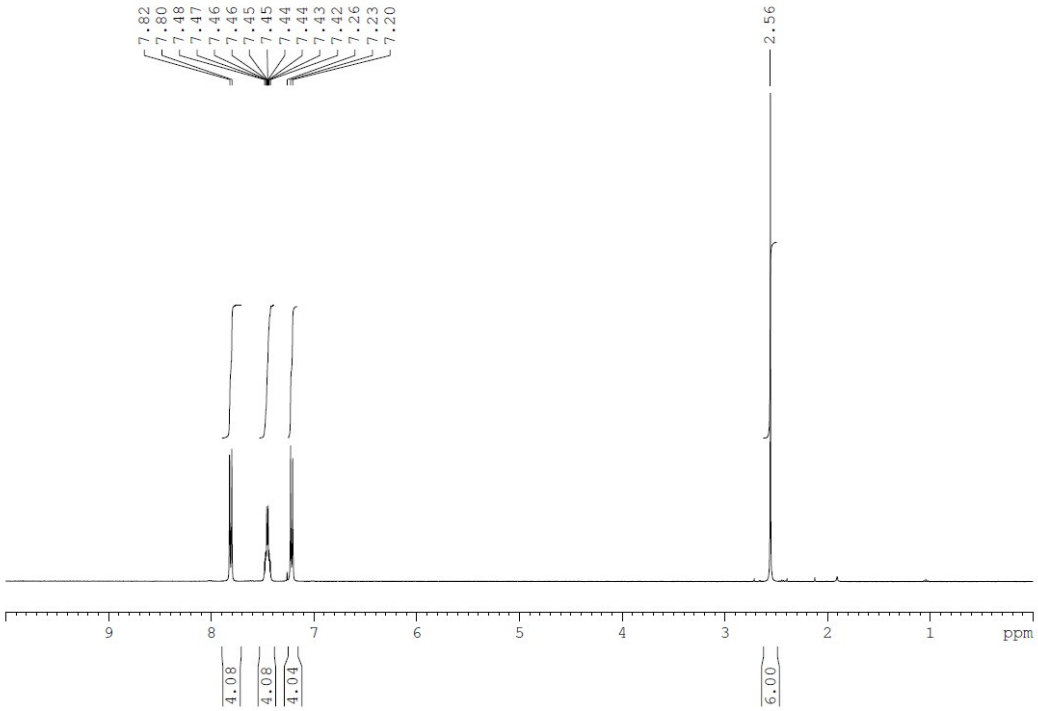


Figure S1: 400 MHz ^{1}H NMR spectrum of 4,4'-diacetyl-*o*-terphenyl **4**.

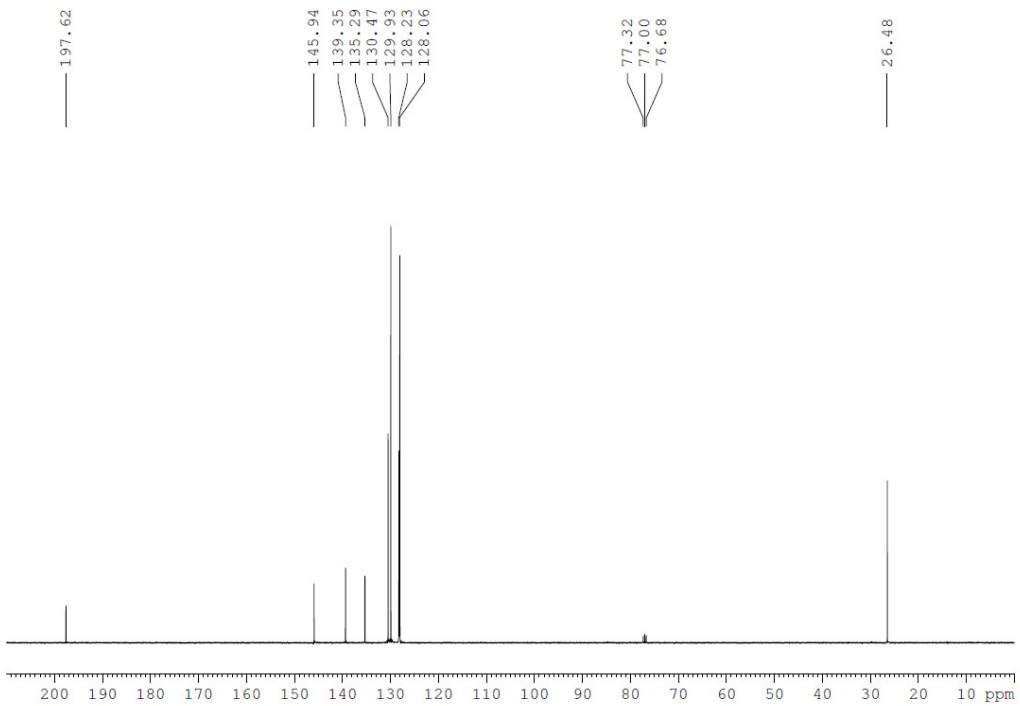
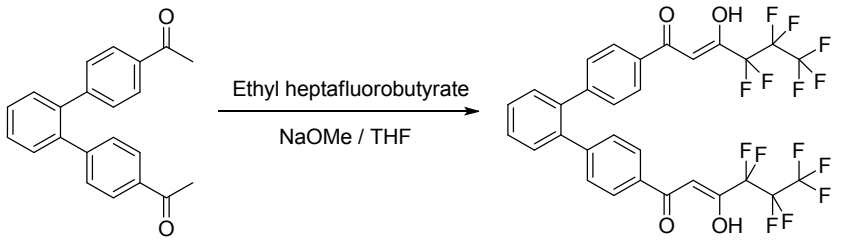


Figure S2: 100 MHz ^{13}C NMR spectrum of 4,4'-diacetyl-*o*-terphenyl **4**.

BHHT 5: (4,4'-Bis-(1",1",1",2",2",2",3",3"-heptafluoro-4",6"-hexandion-6"-yl)-*o*-terphenyl)



Sodium methoxide (520 mg, 9.6 mmol) was added to a stirred solution of 4,4'-diacetyl-*o*-terphenyl **4** (1.0 g, 3.2 mmol) in dry tetrahydrofuran (30 mL) at room temperature under an atmosphere of nitrogen. The reaction mixture quickly turned to a light cloudy orange colour. Ethyl heptafluorobutyrate (1.4 mL, 8.0 mmol) was added drop wise *via* syringe over 10 min while the reaction mixture was stirring. TLC analysis of the reaction mixture after 16 h using [ethyl acetate/hexane (4/1) with one drop of trifluoroacetic acid in a 5 mL mixture] indicated the presence of starting material which was consumed over the next 4 h. The organic solvent was removed under reduced pressure, and the residue was dissolved in ether (50 mL) and quenched with hydrochloric acid (3 M, 20 mL), the ether layer was then dried over anhydrous magnesium sulfate, filtered and evaporated under reduced pressure to give a pale yellow oil that was crystallised in absolute ethanol (5 mL) and collected by filtration to give BHHT **5** (1.9 g, 85%) as a yellow powder. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.58 (s, 2H), 7.29 (d, J = 8.5 Hz, 4H), 7.46-7.55 (m, 4H), 7.85 (d, J = 8.5 Hz, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 93.8, 127.6, 128.8, 130.5, 130.7, 131.2, 139.1, 147.2, 179.0, 185.0. The data are in agreement with those in the literature.[2, 3]

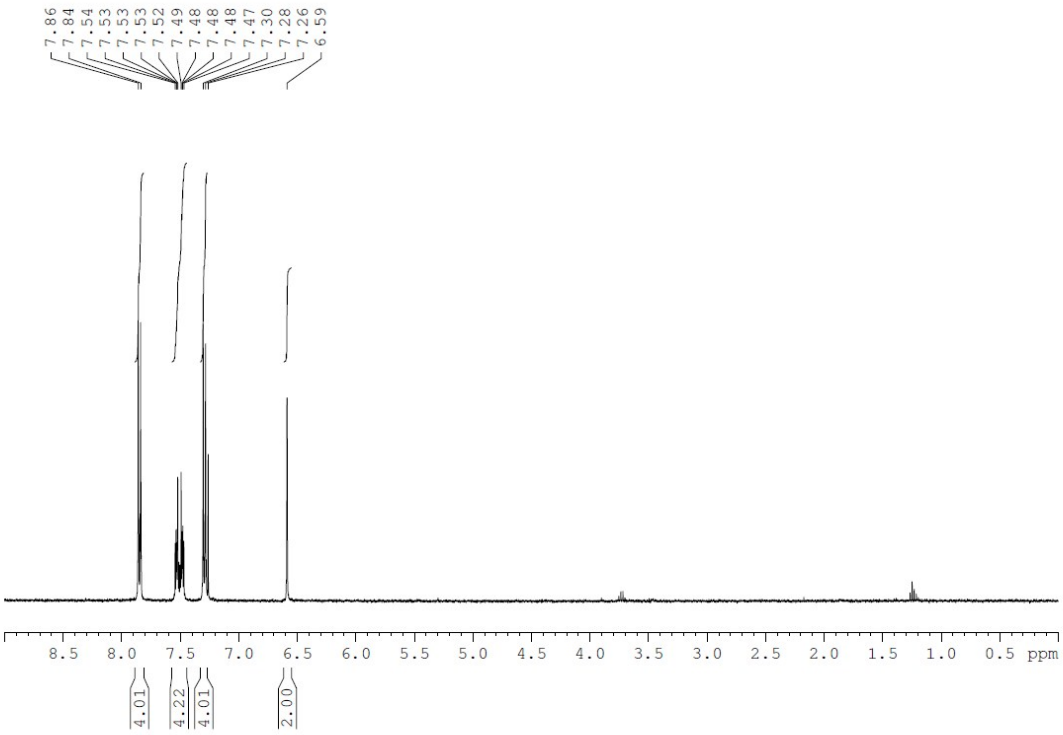


Figure S3: 400 MHz ^{1}H NMR spectrum of BHHT **5**.

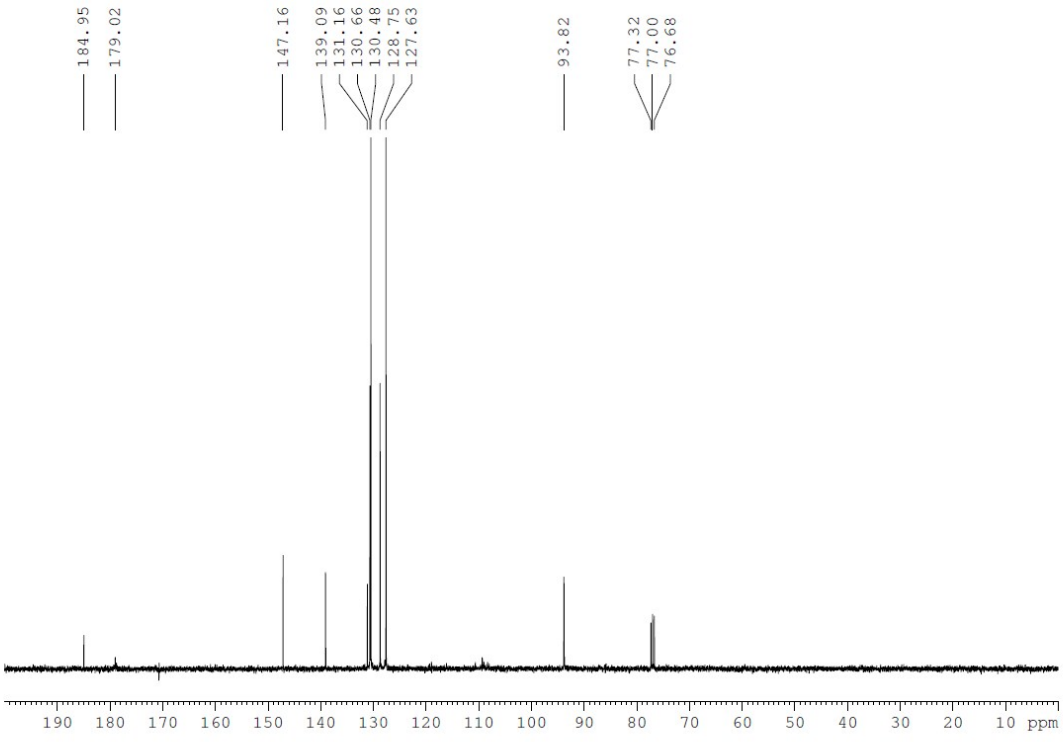
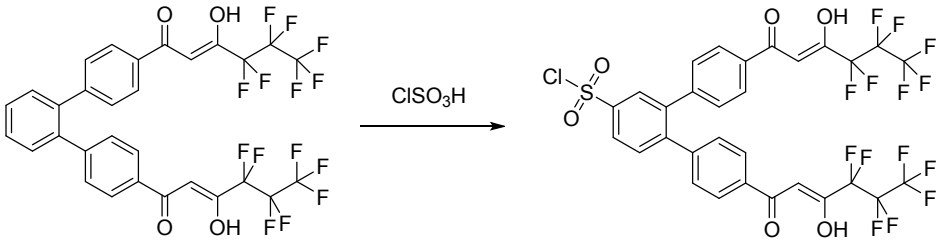


Figure S4: 100 MHz ^{13}C NMR spectrum of BHHT **5**.

BHHCT 2: 4,4''-Bis-(4,4,5,5,6,6,6-heptafluoro-3-oxo-hexanoyl)-[1,1';2',1'']-*o*-terphenyl-4'-sulfonyl chloride)



A 25 ml round bottom flask was dried at 180 °C for 20 min and then charged with chlorosulfonic acid (5 mL). The flask was equipped with a stirrer bar and small portions of BHHT **5** (600 mg; 0.9 mmol) were added slowly. The reaction was allowed to proceed at room temp (4 h) whereon the reaction mix was quenched by drop wise addition to crushed ice. The ice\water mix was then extracted with ethyl acetate (3 x 60 mL), dried over anhydrous sodium sulfate, filtered and concentrated to give crude BHHCT **2** (700 mg, 95%) that was used in future steps without further purification (based on NMR results it was more than 98% pure). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.58 (app s, 2H), 7.30-7.33 (m, 4H), 7.72 (d, *J* = 8.3 Hz, 1H), 7.88-7.91 (m, 4H), 8.13 (d, *J* = 2.0 Hz, 1H), 8.17 (dd, *J* = 2.0, 8.3 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 94.0, 126.7, 127.9, 128.0, 128.8, 130.2, 130.3, 132.0, 132.4, 132.6, 140.9, 144.1, 144.2, 145.9, 184.1, 184.2. The data are in agreement with those in the literature.[2, 3]

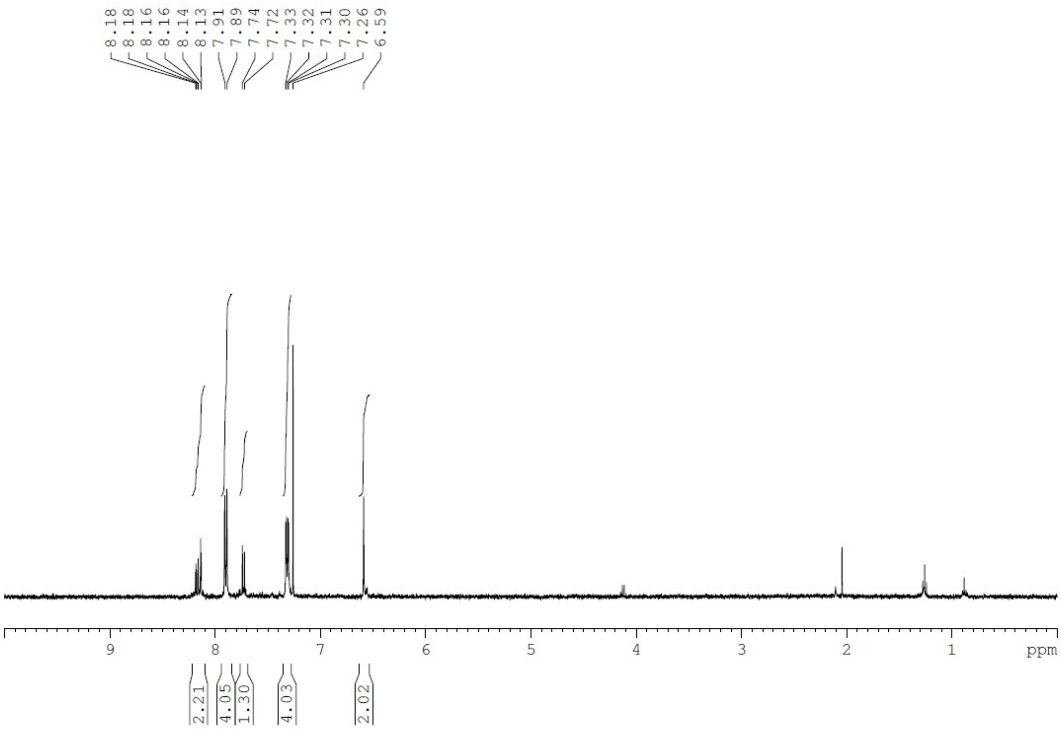


Figure S5: 400 MHz ^{1}H NMR spectrum of BHHCT **2**.

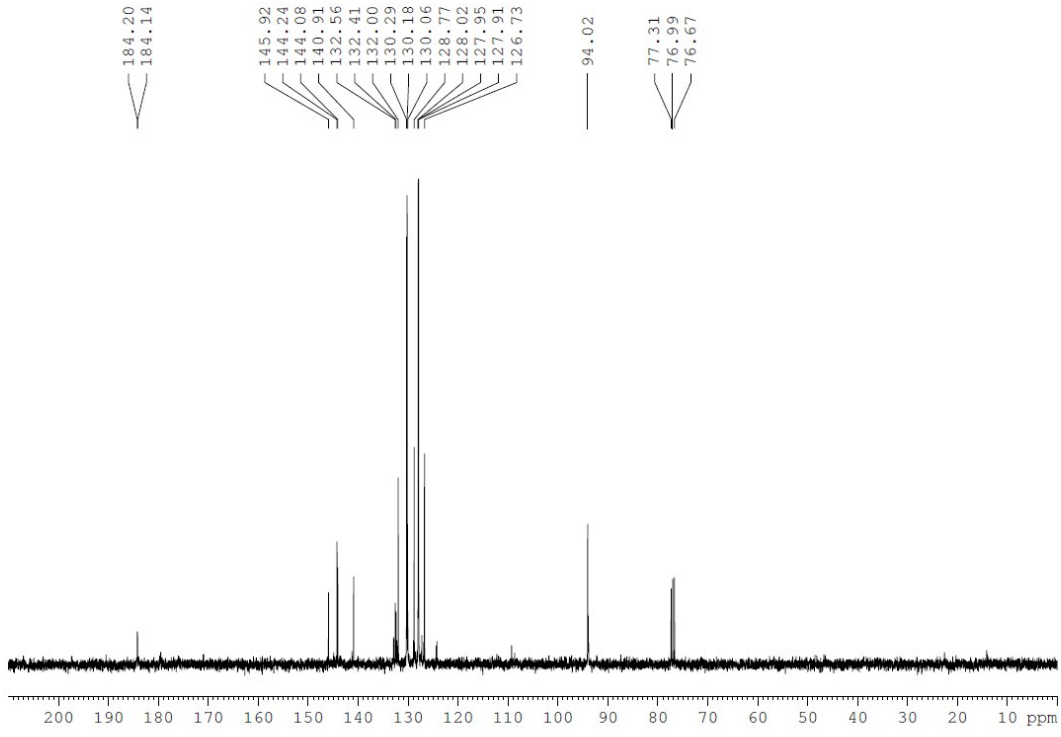
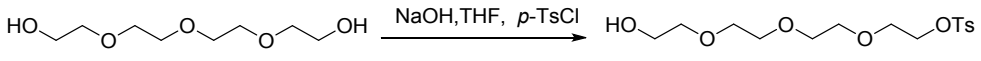


Figure S6: 100 MHz ^{13}C NMR spectrum of BHHCT **2**.

2-[2-[2-(2-Hydroxyethoxy)ethoxy]ethoxy]ethyl 4-methylbenzenesulfonate



Sodium hydroxide (0.69 g, 17.13 mmol) was ground to a coarse powder and quickly added to a solution of tetraethylene glycol (21.95 g, 113 mmol) in tetrahydrofuran (5 mL) at 0 °C, followed by a slow addition of a solution of *p*-toluenesulfonylchloride (2.08 g, 10.93 mmol) in tetrahydrofuran (20 mL). The reaction mixture was then stirred for 2 h at 0 °C and poured into a mixture of ice and water. The organic layer was separated, and the aqueous layer extracted with dichloromethane (3 x 100 mL). The combined organic layers were washed with water (2 x 50 mL), dried over anhydrous magnesium sulfate, filtered and evaporated *in vacuo* to yield 2-[2-[2-(2-hydroxyethoxy)ethoxy]ethoxy]ethyl 4-methylbenzenesulfonate (3.29 g, 86%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.42 (s, 3H), 3.58-3.63 (m, 14H), 4.10-4.15 (m, 2H), 7.32 (d, J = 8.0 Hz, 2H), 7.77 (d, J = 8.0 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 21.6, 61.7, 68.7, 69.2, 70.3, 70.4, 70.6, 70.7, 72.4, 127.9, 129.8, 133.0, 144.8. The data are in agreement with those in the literature.[4]

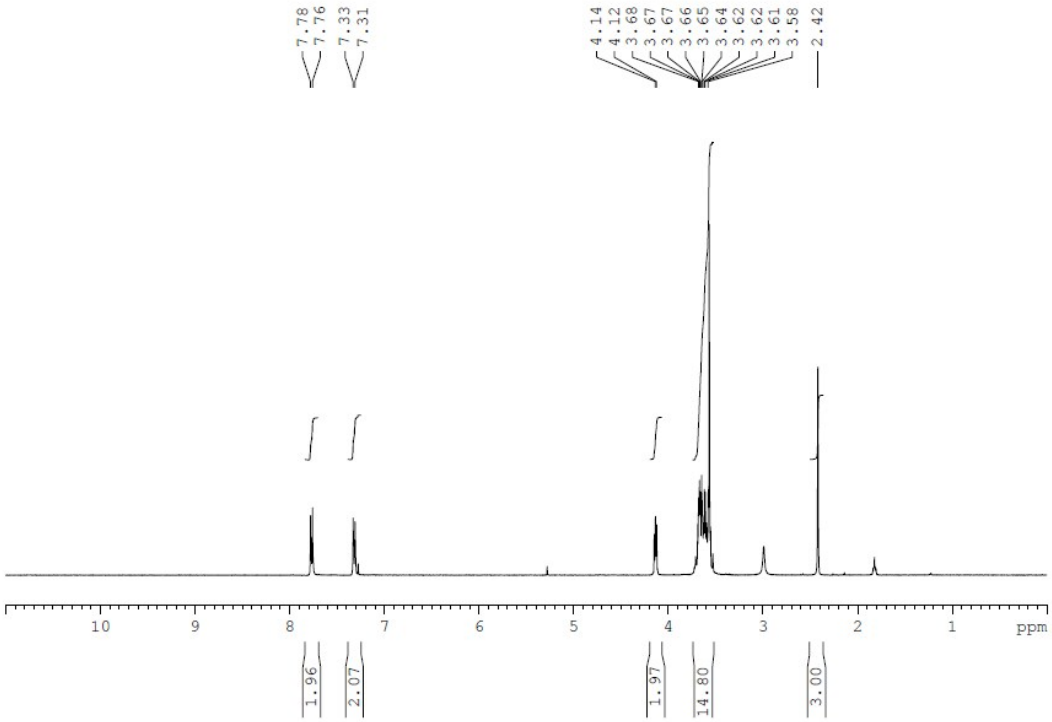


Figure S7: 400 MHz ^{1}H NMR spectrum of 2-[2-[2-(2-hydroxyethoxy)ethoxy]ethoxy]ethyl 4-methylbenzenesulfonate.

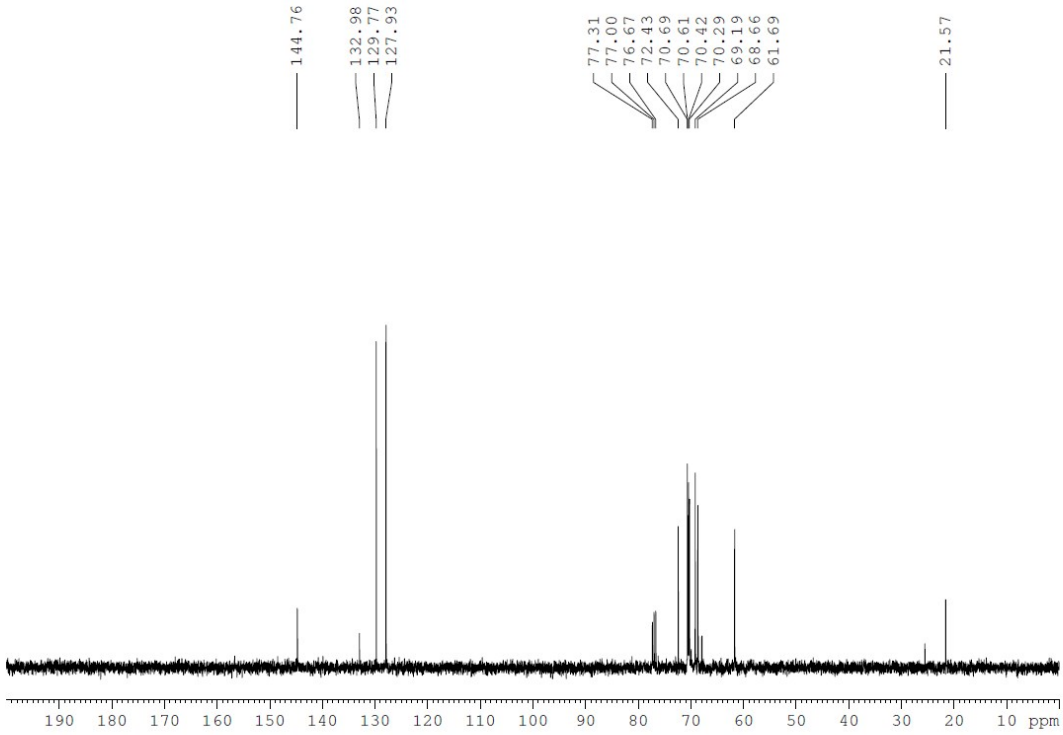
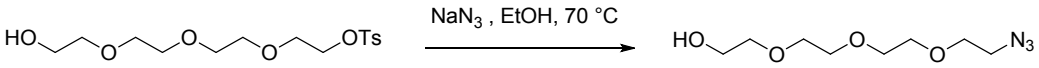


Figure S8: 100 MHz ^{13}C NMR spectrum of 2-[2-[2-(2-hydroxyethoxy)ethoxy]ethoxy]ethyl 4-methylbenzenesulfonate.

2-[2-[2-(2-Azidoethoxy)ethoxy]ethoxy]ethanol



Sodium azide (3.0 g, 46.15 mmol) was added to a solution of 2-[2-[2-(2-hydroxyethoxy)ethoxy]ethoxy]ethyl 4-methylbenzenesulfonate (3.2 g, 9.18 mmol) in ethanol (50 mL) at room temperature. The reaction mixture was stirred overnight at 70 °C. The reaction was then quenched by the addition of water (50 mL) and concentrated under vacuum to a third of its volume. The aqueous layer was extracted with ethyl acetate (3 x 50 mL) and the combined organic layers were then dried over anhydrous magnesium sulfate, filtered and concentrated under vacuum. The residue was then purified by flash column chromatography on silica gel using a mixture of acetone and hexane (2:3) to yield 2-[2-[2-(2-azidoethoxy)ethoxy]ethoxy]ethanol (1.63 g, 81%) as an oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.39 (app t, J = 5.0 Hz, 1H), 3.59-3.63 (m, 2H), 3.65-3.69 (m, 10H), 3.71-3.74 (m, 2H), – OH not observed; ^{13}C NMR (100 MHz, $CDCl_3$) δ 50.6, 61.7, 70.0, 70.3, 70.5, 70.6, 70.7, 72.5. The data are in agreement with those in the literature.[4]

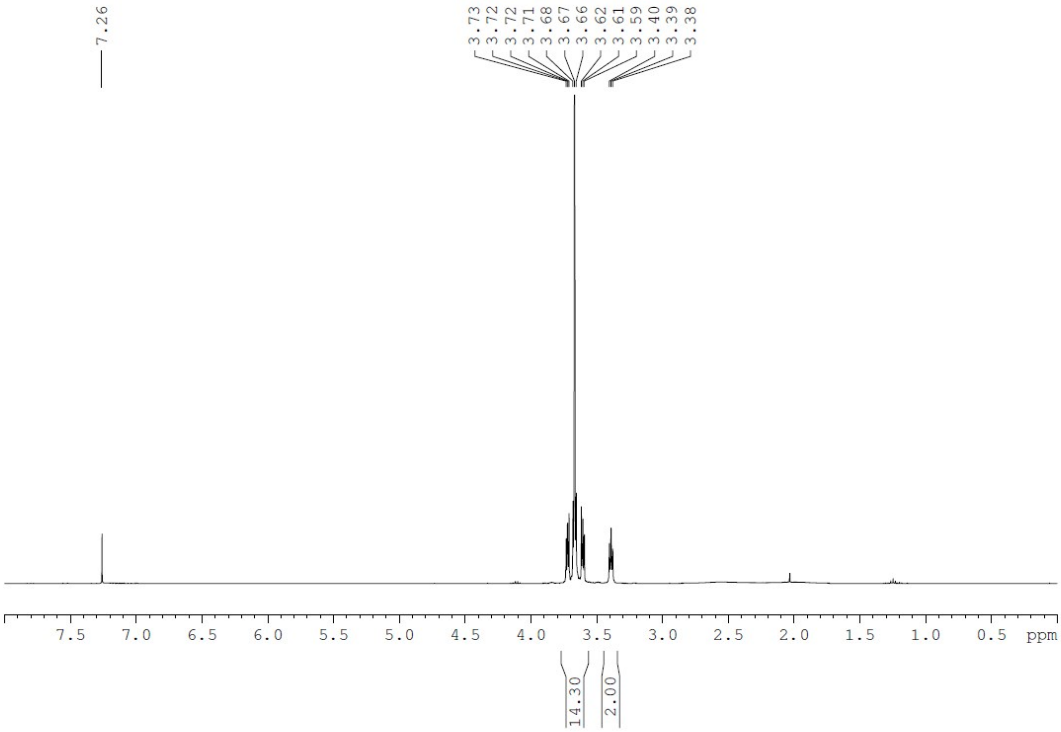


Figure S9: 400 MHz ^{1}H NMR spectrum of 2-[2-[2-(2-azidoethoxy)ethoxy]ethoxy]ethanol.

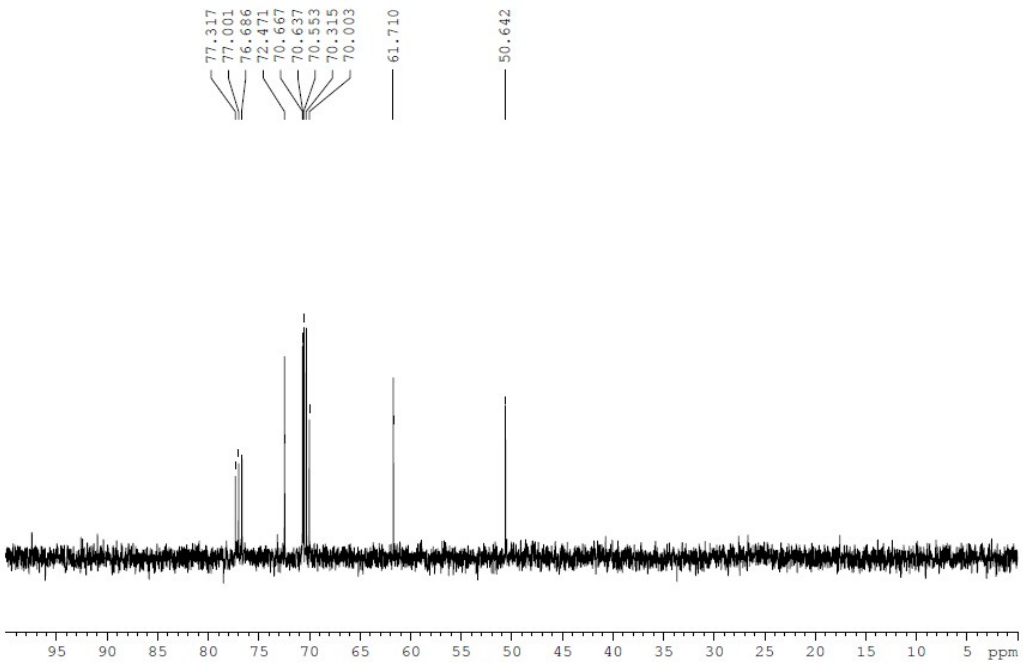
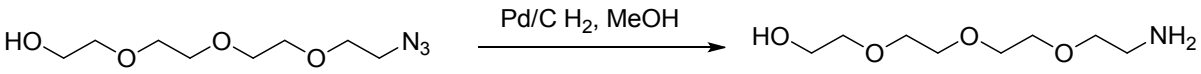


Figure S10: 100 MHz ^{13}C NMR spectrum of 2-[2-[2-(2-azidoethoxy)ethoxy]ethoxy]ethanol.

2-[2-[2-(2-aminoethoxy)ethoxy]ethoxy] ethanol 6



Palladium on carbon (10%, 0.1 g) was added to a solution of 2-[2-[2-(2-azidoethoxy)ethoxy]ethoxy] ethanol (600 mg, 2.7 mmol) in methanol (20 mL). The flask was repeatedly evacuated and then filled with hydrogen gas three times, then the mixture was stirred under a hydrogen atmosphere for 6 h. The mixture was then filtered through a pad of celite, and the celite washed with methanol (2 x 50 mL). The filtrates were combined and the solvent removed under reduced pressure to give 2-[2-[2-(2-aminoethoxy)ethoxy]ethoxy] ethanol **6** (495 mg, 95%) as a colourless oil which was used without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.77 (app t, J = 5.1 Hz, 2H), 3.49-3.70 (m, 14H), - OH not observed; ^{13}C NMR (100 MHz, $CDCl_3$) δ 48.9, 61.4, 70.0, 70.17, 70.20, 70.4, 70.5, 72.9; ESI-MS (positive mode) m/z = 194 ($[M+H]^+$, 100 %); calculated for $C_8H_{19}NO_4$: 194.1. The data are in agreement with those in the literature.[5]

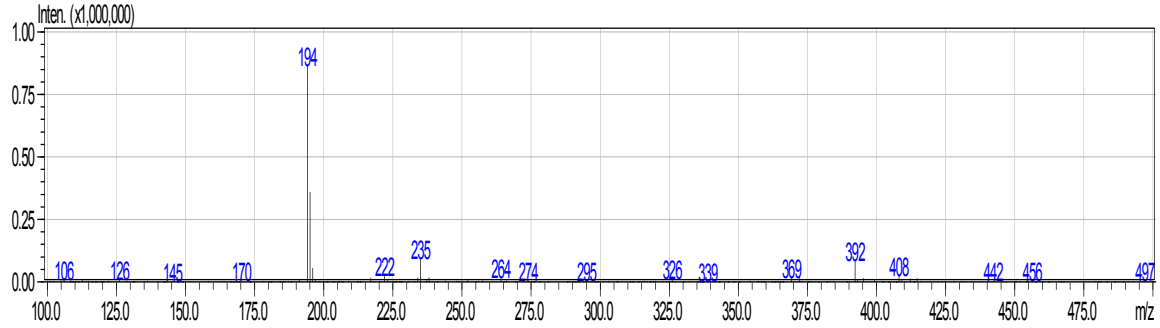


Figure S11: ESI-MS of 2-[2-[2-(2-aminoethoxy)ethoxy]ethoxy]ethanol **6** (positive mode): m/z = 194 ($[M+H]^+$).

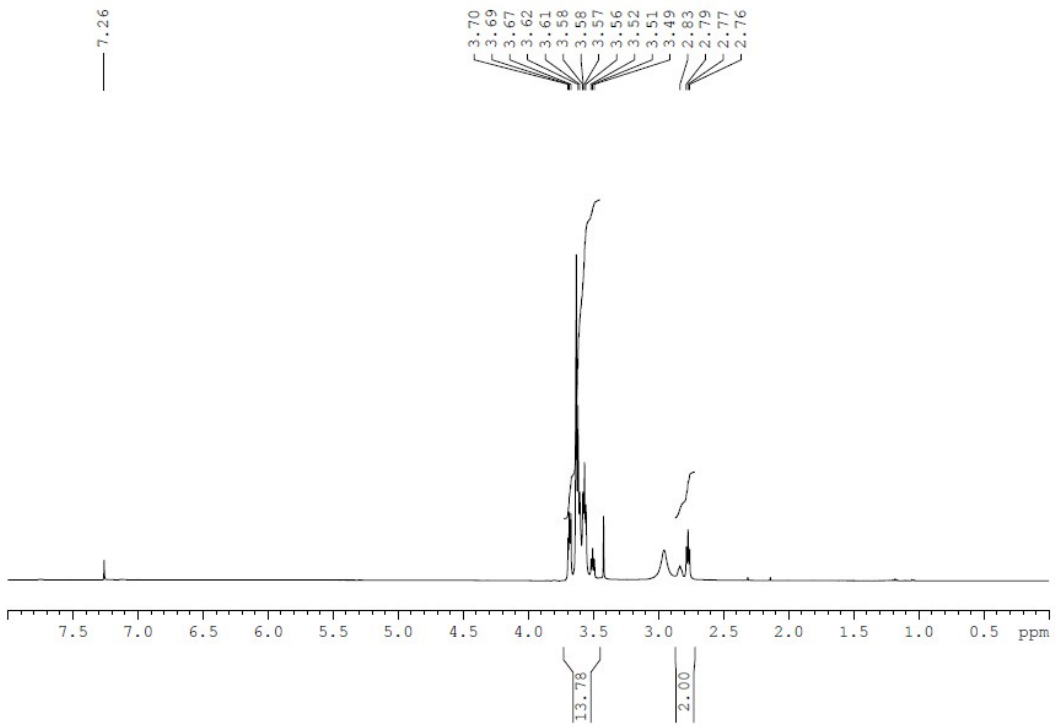


Figure S12: 400 MHz ^{1}H NMR spectrum of 2-[2-[2-(2-aminoethoxy)ethoxy]ethoxy]ethanol **6**.

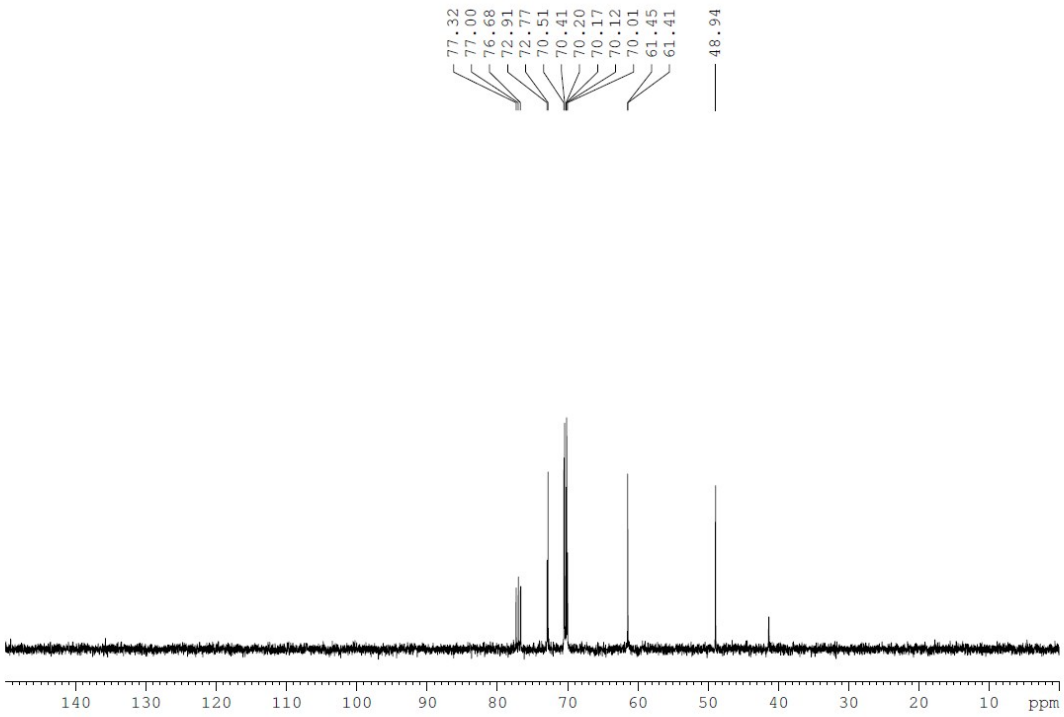
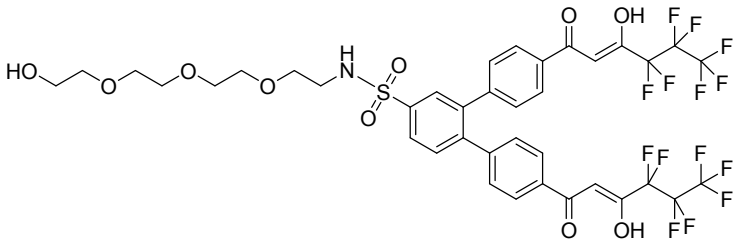


Figure S13: 100 MHz ^{13}C NMR spectrum of 2-[2-[2-(2-aminoethoxy)ethoxy]ethoxy]ethanol **6**.

BHHCT-TEG-OH 7



A stirred solution of 2-[2-[2-(2-aminoethoxy)ethoxy]ethoxy]ethanol **6** (215 mg, 1.1 mmol), *N*,*N*-4-dimethylaminopyridine (24 mg, 0.2 mmol) and triethylamine (145 µL, 1.0 mmol) in dry acetonitrile (10 mL) were combined under anhydrous conditions. Then a solution of BHHCT **2** (800 mg, 1.0 mmol) in dry acetonitrile (5 mL) was added in drop wise *via* a syringe over a 10 min period. The progress of the reaction was monitored by TLC (chloroform:methanol, 9:1 and a drop of trifluoroacetic acid) which indicated completion of reaction in less than 1 h (BHHCT **2** $R_f = 0.5$, BHHCT-TEG-OH **7** $R_f = 0.45$). Acetonitrile was removed under reduced pressure at 45 °C and the residue was partitioned between ethyl acetate (50 mL) and an aqueous solution of potassium hydrogen sulfate (1 M, 20 mL). The organic layer was washed with potassium hydrogen sulfate (20 mL) and saturated brine (20 mL) and dried over anhydrous magnesium sulfate, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography (chloroform:methanol, 9:1 and 0.01% trifluoroacetic acid) to give BHHCT-TEG-OH **7** (800 mg, >80%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.20 (app t, 2H), 3.56-3.67 (m, 10H), 3.69-3.73 (m, 2H), 3.75-3.78 (m, 2H), 6.58 (app s, 2H), 7.27-7.32 (m, 4H), 7.59 (d, J = 7.5 Hz, 1H), 7.83-7.88 (m, 4H), 7.98-8.03 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 43.1, 61.4, 69.7, 69.9, 70.1, 70.6, 72.4, 93.9, 126.9, 127.7, 129.0, 130.3, 130.4, 131.3, 131.8, 131.9, 139.9, 140.9, 142.8, 145.3, 145.4, 184.46, 184.52; ESI-MS (negative mode) m/z = 960 [M^+−H], 100 %); HRMS (ESI): calculated for $C_{38}H_{33}F_{14}NO_{10}SNa$: 984.1499, Found 984.1483.

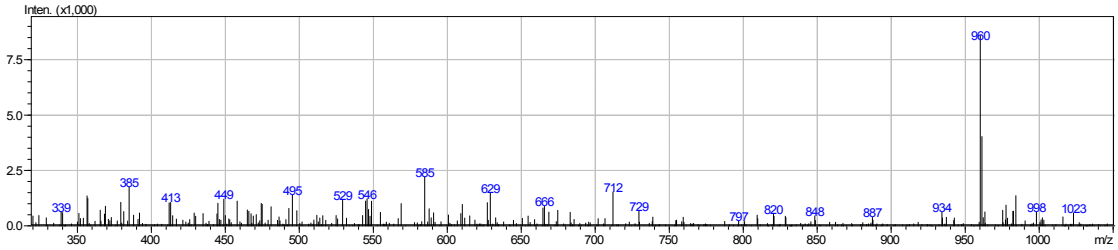


Figure S14: ESI-MS of BHHCT-TEG-OH **7** (negative mode): m/z = 960 ([M-H]$^-$).

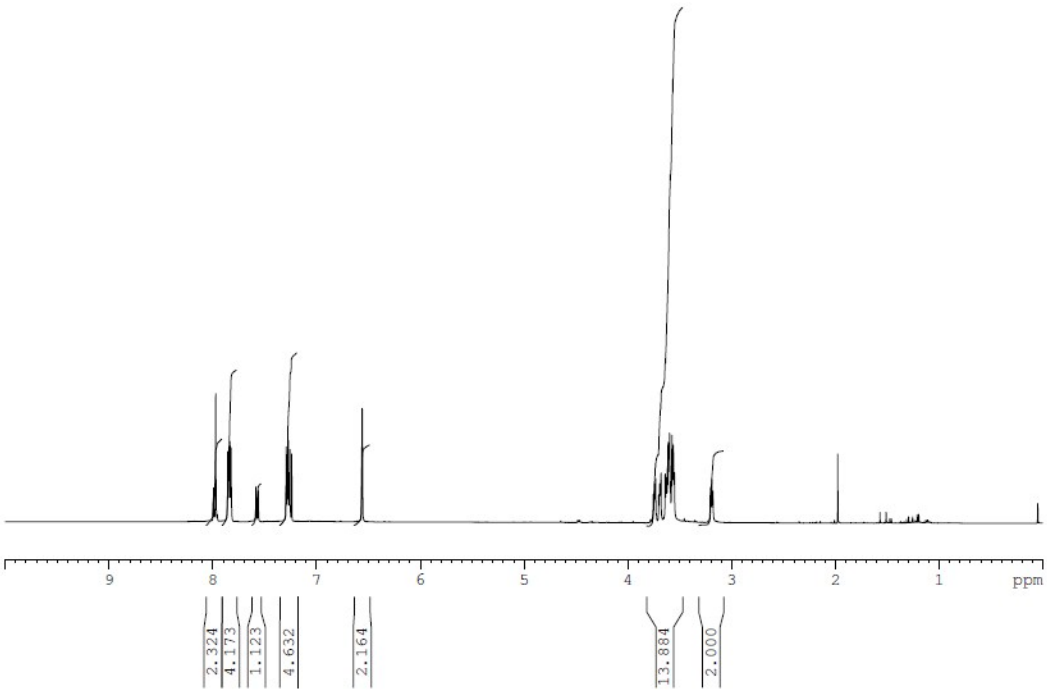


Figure S15: 400 MHz ^{1}H NMR spectrum of BHHCT-TEG-OH **7**.

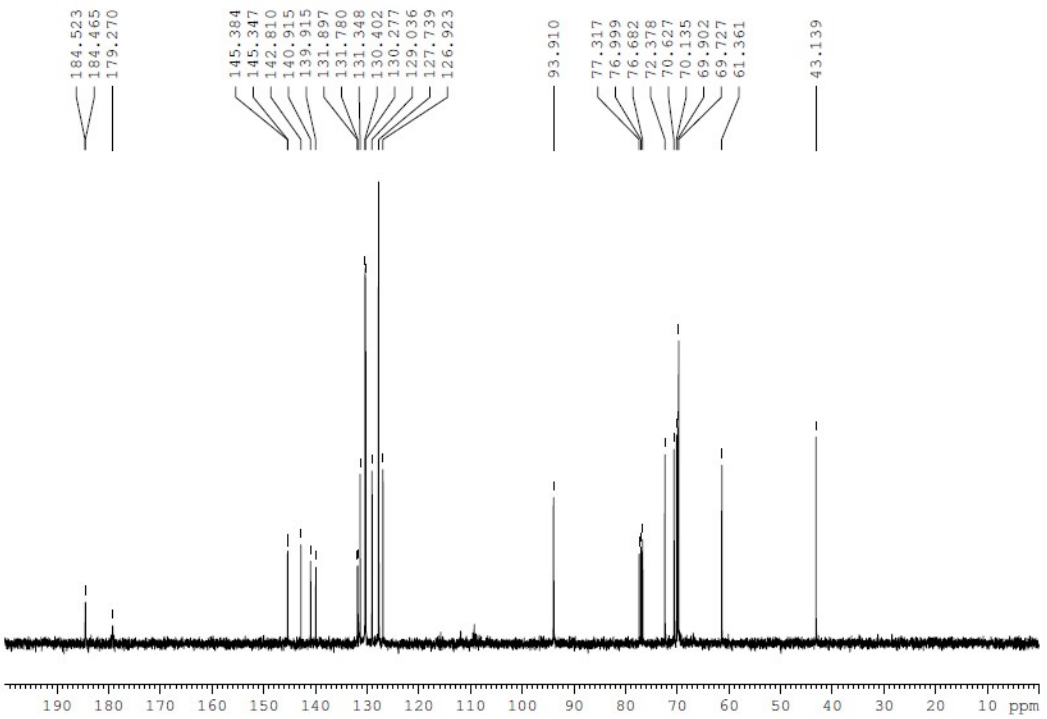
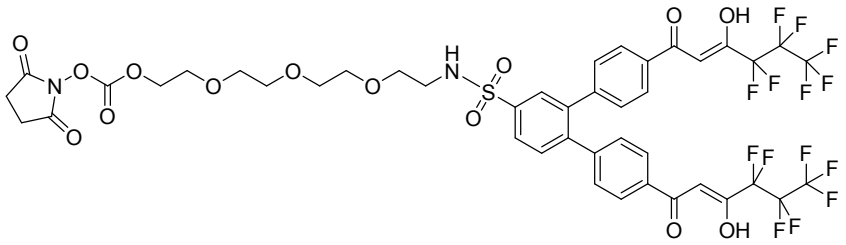


Figure S16: 100 MHz ^{13}C NMR spectrum of BHHCT-TEG-OH **7**.

BHHTEGST 1: 4,4' –Bis(1",1",1",2",2",3",3"-heptafluoro-4",6"-hexanedion-6"-yl) sulfonylamino-tetraethyleneglycol-succinimidylcarbonate-*o*-terphenyl



To a stirred solution of BHHCT-TEG-OH **7** (125 mg, 0.13 mmol), 4-dimethylaminopyridine (DMAP) (12 mg, 0.1 mmol) and triethylamine (50 µL, 0.4 mmol) in dry acetonitrile (10 mL) under anhydrous conditions was added *N*,*N*-disuccinimidyl carbonate (100 mg, 0.4 mol). The mixture was then stirred for 3 h at room temperature, after which time TLC analysis of the reaction mixture indicated the completion of reaction (chloroform:methanol, 9:1 and 0.01% trifluoroacetic acid) (BHHCT-TEG **7**; R_f = 0.45, BHHTEGST **1**; R_f = 0.55). Solvent (acetonitrile) was removed under reduced pressure at 45 °C and the residue was purified by flash column chromatography (chloroform:methanol, 9:1 and a drop of trifluoroacetic acid) to give BHHTEGST **1** as a light yellow powder (100 mg, 70%). HPLC of this partially purified material afforded pure BHHTEGST **1** as a light yellow lyophilized powder (92 mg, 65% yield based on **7**). ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.84 (s, 4H), 3.20-3.26 (m, 2H), 3.58-3.70 (m, 10H), 3.75-3.80 (m, 2H), 4.42-4.48 (m, 2H), 6.58-6.60 (m, 2H), 7.27-7.33 (m, 4H), 7.62 (d, *J* = 7.5 Hz, 1H), 7.85-7.89 (m, 4H), 7.97-8.02 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 25.4, 43.2, 61.3, 68.3, 68.7, 69.3, 70.2, 70.3, 70.5, 70.6, 70.9, 93.9, 127.0, 127.8, 129.0, 130.3, 130.4, 130.5, 131.5, 140.0, 140.6, 143.0, 145.3, 168.7, 184.5; ESI-MS (negative mode) m/z = 1101 ([M^+−H], 100%); HRMS (ESI): calculated for $C_{43}H_{36}F_{14}N_2O_{14}SNa$ $(M+Na)^+$: 1125.1561, Found 1125.1545.

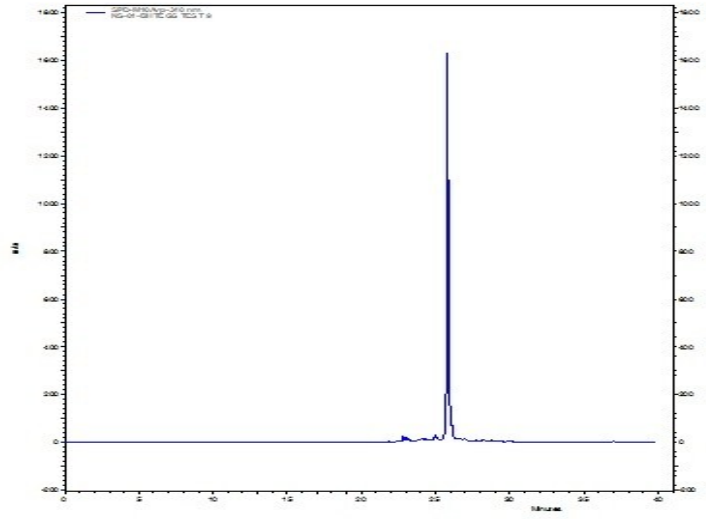


Figure S17: Analytical HPLC chromatogram of BHHTEGST **1** (retention time 22.3 mins).

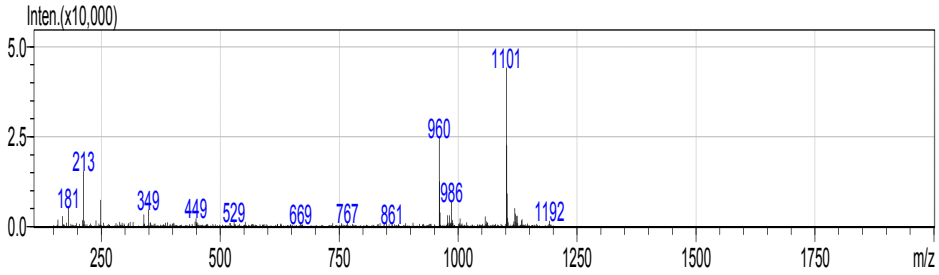


Figure S18: ESI-MS (negative mode) spectrum of BHHTEGST **1**.

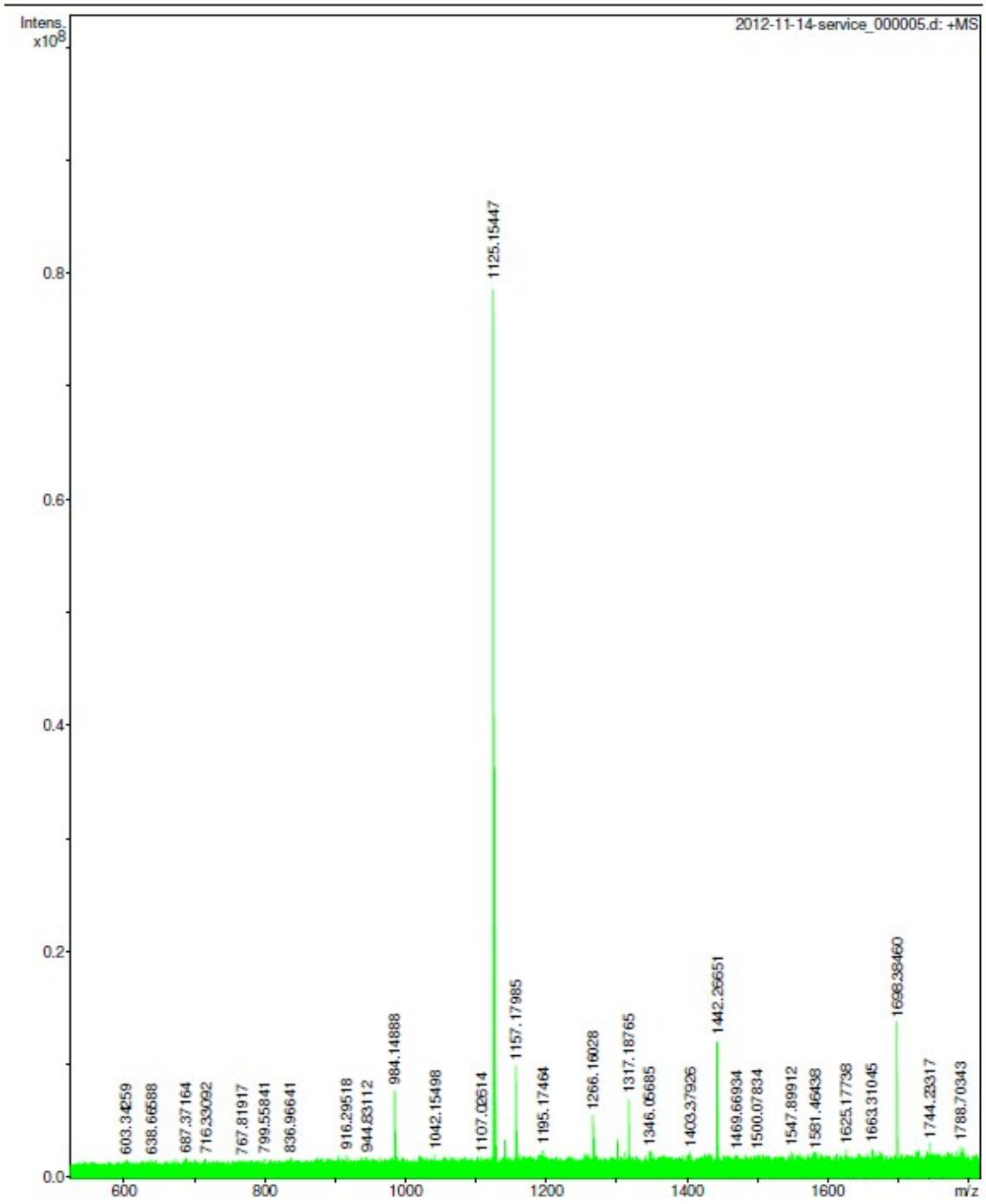


Figure S19: High resolution mass spectrometry (HRMS) [positive mode] of BHHTEGST **1**.

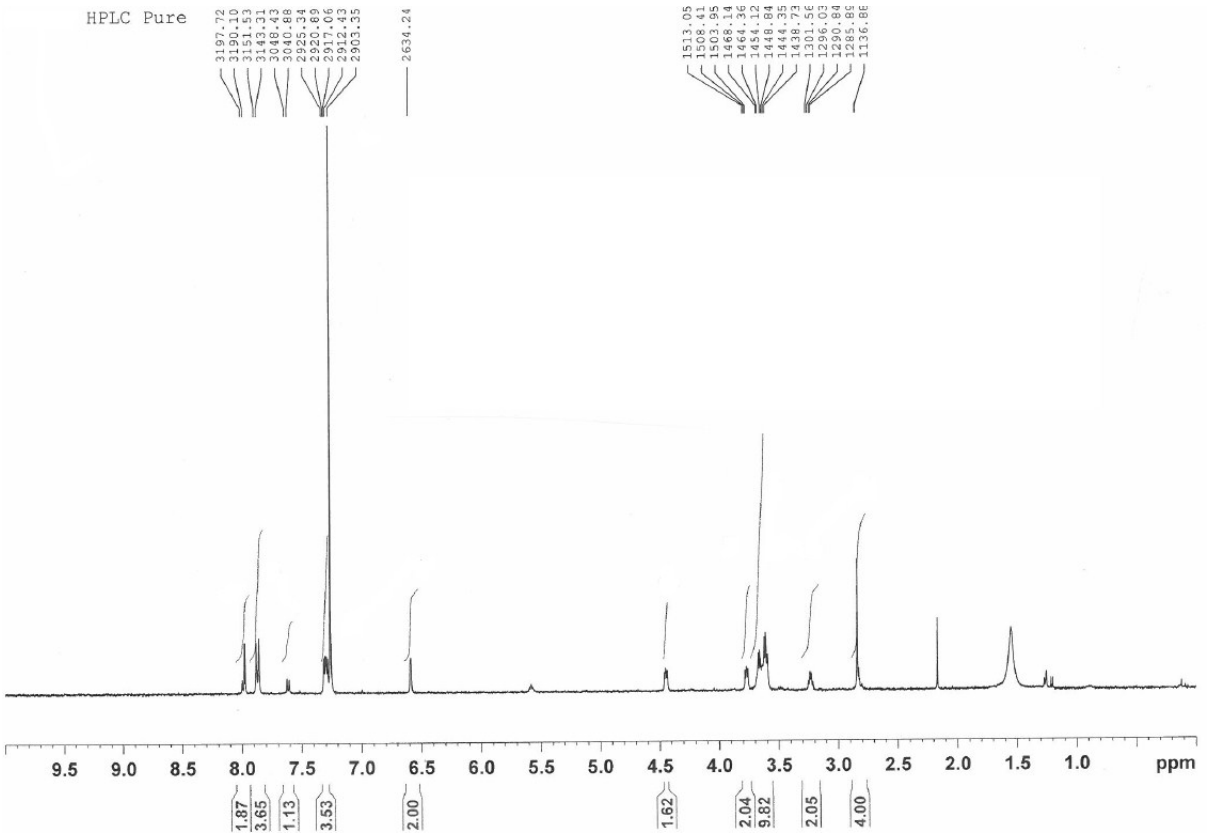


Figure S20: 400 MHz ^{1}H NMR spectrum of BHHTEGST **1**.

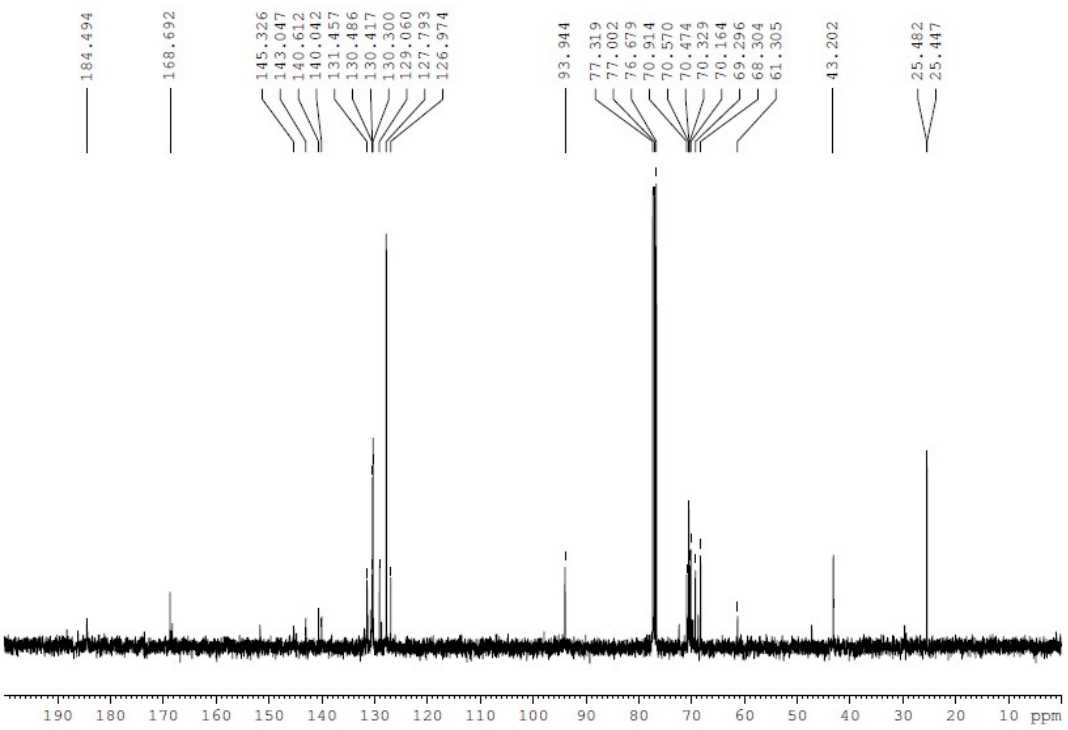


Figure S21: 100 MHz ^{13}C NMR spectrum of BHHTEGST **1**.

Conjugation of antibody with BHHTEGST

BHHTEGST contains an *N*-hydroxysuccinimide ester that enables its attachment to antibody *via* the amino group of lysine residues. In the conjugation reaction, 100 μg antibody was exchanged into 100 mM $NaHCO_3$, pH 8.5 and then mixed with different molar excess of the BHHTEGST ligand, see Table S1. After incubation for 1 h at 37 °C the reaction mixtures were passed through a Sephadex G-50 column in 0.1 PBS to remove excess BHHTEGST **1**. The fractions corresponding to labeled conjugates were detected by using a spectrophotometer (280 nm and 335 nm).

Standard cell-labelling procedure

Cell fixation was carried out by gently drying a 10 μl cell sample on a slide with a hot plate at 40 °C for 20 s. To stain the *Giardia cysts*, 3 μl of conjugated G2O3 with BHHTEGST (1-10 μg/mL) and 3 μl G2O3-FITC (1-10 μg/mL) were mixed and loaded onto a microscope slide containing fixed target cells and incubated for 5 min at room temperature. The slide was gently rinsed with MQ water and then 3 μL europium chloride [$EuCl_3$, 2 mM in 1X Fluorescence Enhancing Buffer (FEB) see page S35] and 2 μL of DAPI (2 μg/ml in PBS) were added to the slide and allowed to react with the ligands for 2 minutes. The labelled cells were then examined using bright-field, FITC and time-gated luminescence microscopy.

Imaging results of *Giardia cysts* staining and SNR quantification

The images are original as taken, no post processing was performed; Signal to Noise ratios (SNR) were calculated using ImageJ software version 1.46r. Representative images for conjugates were captured and analyzed to determine the SNR as illustrated in Figure S24 & 25. For our purposes, signal is represented by the brightest region of desired target signal (S) and noise is the mean non-target signal (N). ImageJ is used to measure peak signal intensity (S) of an area in the target cell and noise is the mean intensity of pixels defined by an area in a non-cell containing area.

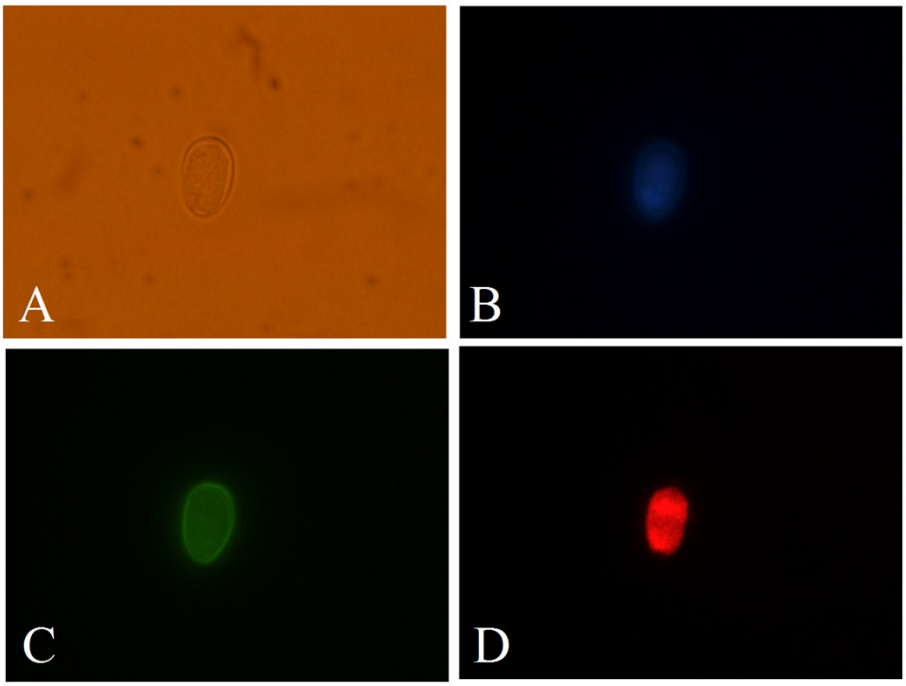


Figure S22. Double labelling of *Giardia cyst* cells using a mix of (G2O3-BHHTEGST-Eu and G2O3-FITC) (A) Bright-field (B) DAPI (C) FITC (D) time-gated condition [GALD].

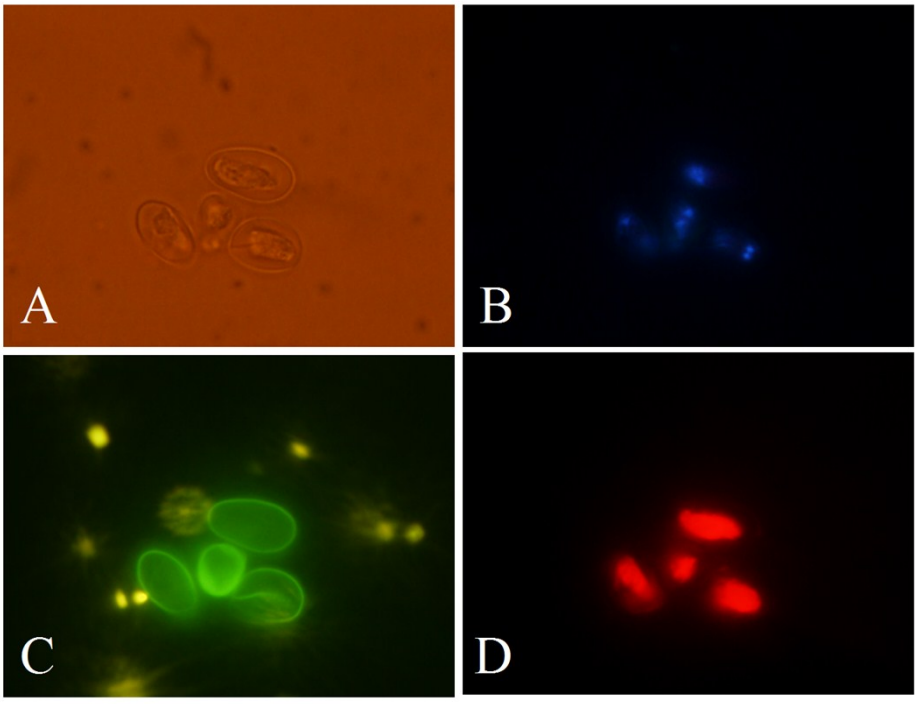


Figure S23. Double labelling of *Giardia cyst* cells using a mix of (G2O3-BHHTEGST-Eu and G2O3-FITC) (A) Bright-field (B) DAPI (C) FITC (D) time-gated condition [GALD] [environmental stimuli background fluorescence (*Synechococcus* cells)].

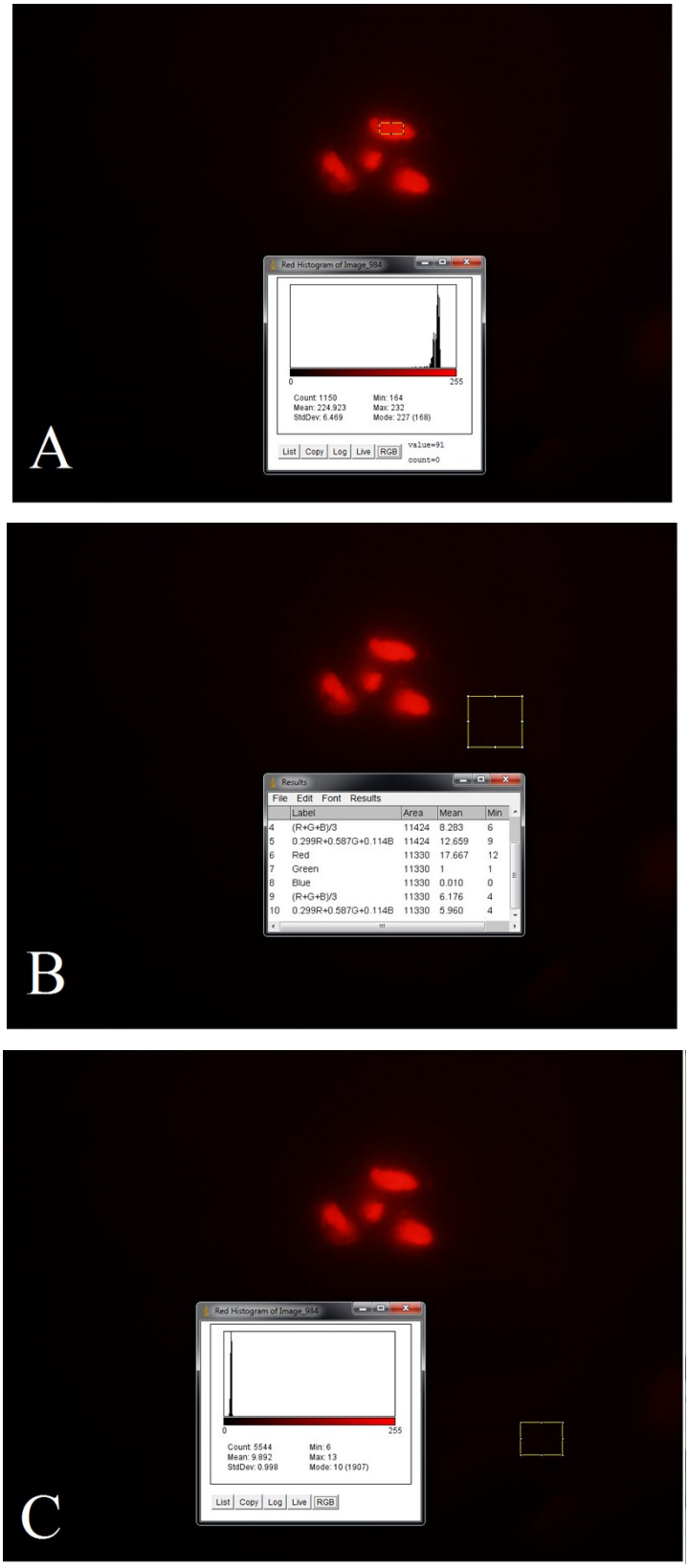


Figure S24. Quantification of signal to noise ratio in europium labelled *Giardia cyst* cells under time-gated condition [GALD]. (A) Signal 225 [mean brightness in cell] (B) Background 17.7 [mean red channel] (C) Background 9.9 [mean lowest region of background].
Signal to noise ratio (SNR) calculated to be 12.7 and 22.7 for lowest region of background.

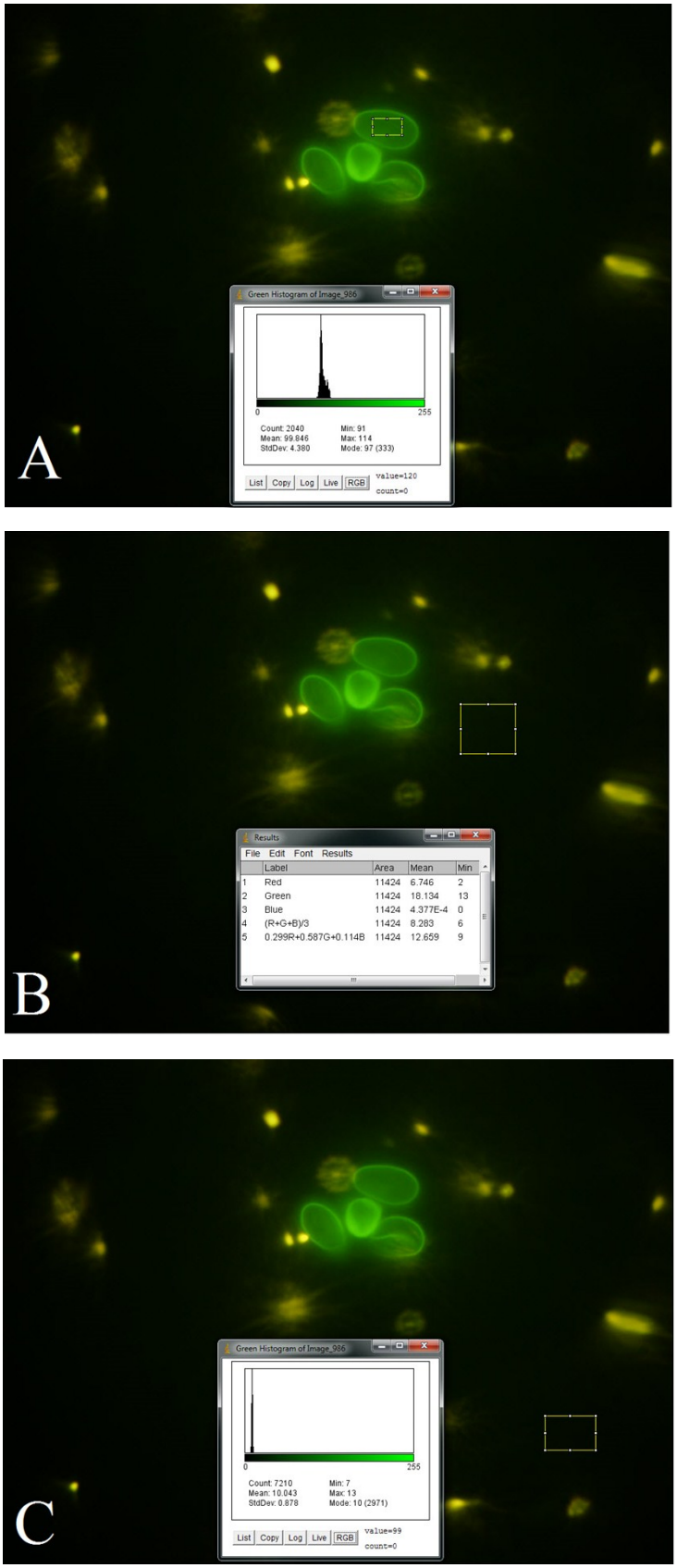


Figure S25. Quantification of signal to noise ratio in FITC labelled *Giardia cyst* cells under FITC channel (A) Signal 99.8 [mean brightness in cell] (B) Background 12.6 [mean green, red & blue channel] (C) Background 10 [mean lowest region of background]
Signal to noise ratio (SNR) calculated to be 7.9 and 9.98 for lowest region of background.

Quantification of BHHTEGST ligands attached to antibody

UV-visible absorption analysis of BHHTEGST (NanoDrop UV spectrometer) indicated a maximum UV absorption at 335 nm and also partial absorption at 280 nm which overlaps with that of the antibody G2O3. To evaluate the partial absorption of BHHTEGST moiety in the conjugated antibody; molar extinction coefficient of the ligand at 335 nm and 280 nm was separately obtained from UV-visible analysis of solution of HPLC pure lyophilized ligand at 335 nm and 280 nm, respectively [ε_{335} = 3.14 x 10^4 $M^{-1}.cm^{-1}$, ε_{280} = 1.75 x 10^4 $M^{-1}.cm^{-1}$] (see Table S2). The concentration of ligand was then obtained by reading the absorbance of conjugates at 335 nm and using the known molar extinction coefficient of the ligand at 335 nm (on the assumption that the extinction coefficient of BHHTEGST **1** does not change on the labelled antibody). Partial absorption of ligand at 280 nm was identified by having its concentration and its molar extinction coefficient at 280 nm. Consequently, antibody concentration was obtained by subtracting the absorbance of the ligand from the absorbance of labelled protein at 280 nm. The number of BHHTEGST molecules per antibody then was obtained by dividing the molar ratio of ligand to antibody.

Conjugation ratio (experimental conditions) BHHTEGST : Antibody (Molar excess)	**Number of ligands per Antibody** (calculated ratio after purification) G2O3-BHHTEGST$_X$
20:1	X = 10
40:1	X = 14
60:1	X = 19

Table S1. Quantification of the number of BHHTEGST ligands attached to G2O3 after conjugation reactions were performed with three levels of BHHTEGST molar ratio to antibody of 20:1, 40:1 and 60:1.

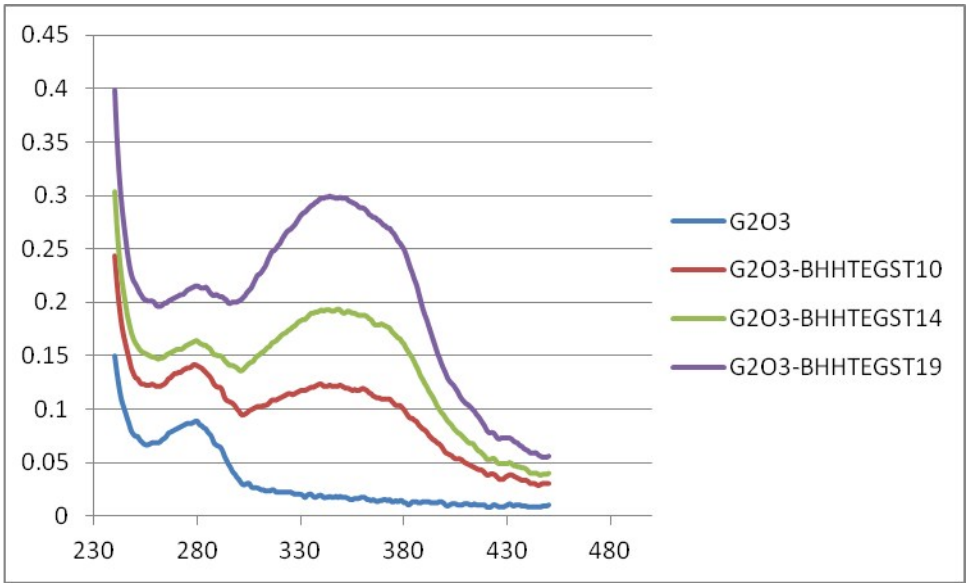


Figure S26: UV-visible spectrum of G2O3 only and the 3 levels of BHHTEGST conjugation with G2O3. The band at 335 nm (from BHHTEGST component absorbance) intensifies with respect to the band at 280 nm (from protein component absorbance) as a result of the number of attached equivalents of BHHTEGST **1** is increased.

Titration of BHHCT and BHHBTEGST with Eu^{+3}

Dilute solutions of BHHTEGST and BHHCT (2.5 μM) were prepared in $NaHCO_3$ buffer (100 mM; pH=8). Luminescence emission at 613 nm was monitored for each chelate as fractional equivalents of europium chloride solution were carefully added over a period of 3 hours and twenty minutes. Luminescence emission was measured (Agilent Cary Eclipse Fluorescence Spectrophotometer) 20 min after each addition and titration curves were obtained by plotting the luminescence intensity against the Eu/BHHCT and Eu/BHTEGST molar ratios respectively.

As shown in Figure S27, the luminescence intensity for both chelates increased until 1 molar ratio of Eu^{+3} to ligand was reached, implying the formation of BHHTEGST-Eu and BHHCT-Eu complexes. Further increasing the concentration of Eu^{+3} ions did not increase luminescence intensity of chelates strongly suggesting that the chelates are saturated at 1:1 ratio with Eu^{+3} even with 10 equivalent molar ration of Eu^{+3} ions to ligand. The formation of stable 1:1 complex of Eu^{+3} ions to ligands is in accordance with a previous study on a similar diketone chelate BTBCT by Feng-Bo Wu *et al*.[6] The luminescence spectrum of each chelate was recorded during the titration as shown in Figures S28 & S29.

Experimental:

1.40 mg HPLC pure lyophilized powder of BHHTEGST and 1.00 mg of BHHCT were accurately weighed (0.01 mg accuracy scale) and dissolved in 50 µL of DMF. Then 5 µL of dissolved ligands were diluted to 500 µL with $NaHCO_3$ buffer (100 mM; pH=8) for each ligand respectively. From this solution 10 µL was added to 1 mL $NaHCO_3$ buffer in a quartz cuvette containing europium chloride [0.1 up to 10 equivalent of ligand (2.5 µM)]. A stock solution of Eu^{+3} was prepared in a 10 mL volumetric flask by dissolving (0.916 mg, 0.25 mM) europium chloride hexahydrate in 100 mM $NaHCO_3$ buffer. Each experiment was incubated for 20 min. at room temperature and then luminescence intensity measured using the Cary fluorescence spectrophotometer.

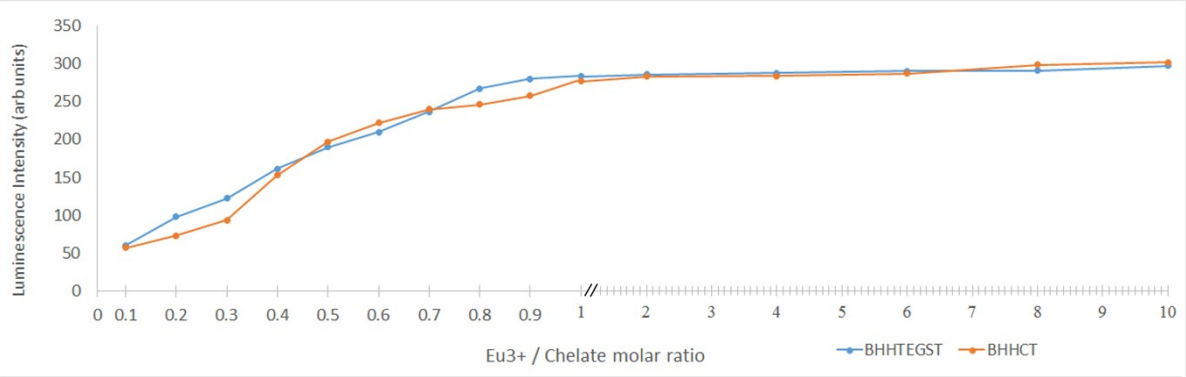


Figure S27: Binding isotherm of luminescence emission intensity ($\lambda_{em\text{-}max}$ = 613 nm) of BHHTEGST-Eu & BHHCT-Eu chelates (2.5 µM) at different molar ratio of Eu^{3+} to ligand (0.1-10 eq.). Emission was seen to peak around 1:1 equivalency of Eu^{3+} ion to ligand. Excited at 335 nm and luminescence emission recorded at 613 nm. (//: After 1:1 ratio of Eu to ligand the units of x axis was changed from 0.1 to 1.0).

UV-visible absorption of BHHCT and BHHTEGST:

UV-visible absorption of the synthesised ligands (BHHCT and BHHTEGST) and also commercially available BHHCT (CAS: 200862-70-0) were investigated using a NanoDrop 2000 UV (Thermo Scientific) spectrometer. All ligands have the same maximum peak absorption at 335 nm as shown in Figure S28. Three replications of each ligand were prepared and the average UV-visible absorption was used to calculate extinction coefficient of ligands at 335 nm as shown in Table S2.

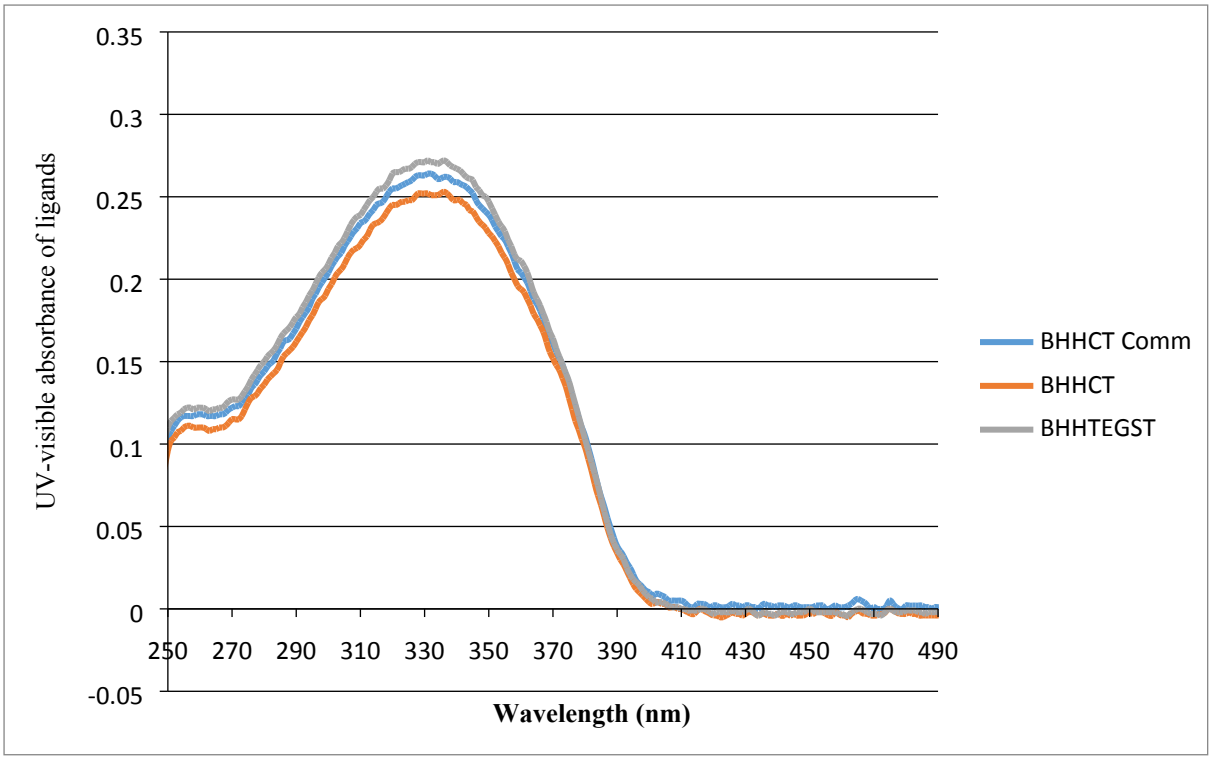


Figure S28: UV-visible profile of BHHCT, BHHCT commercial and BHHTEGST ligands (~86 µM) ($\lambda_{ex.max}$ =335 nm).

Experimental:

From 1.8 / 2.5 / 1.9 mg in 100 µl DMF [BHHCT Comm/BHHCT/BHHTEGST], 7.7/5.5/10 µl was taken and added to 92.3/95/90 µl of DMF to achieve similar molarities and dilution of each ligand. Then from this solution 5.0 µl was added to 95 µl of FEB and analysed on the NanoDrop 2000 UV (Thermo Scientific) spectrometer. Three replications for each ligand were performed and the average reading was then used for calculation of extinction coefficient of ligands at 335 nm.

Ligand	UV-visible Absorption (average of 3 replicates)	M (mmole/mL)	ε_{335} $M^{-1}.cm^{-1}$ (average of 3 replicates)	Cell length cm
BHHCT Commercial	0.261	~ 8.60×10^{-05}	30300	0.1
BHHCT	0.258	~ 8.54×10^{-05}	30200	0.1
BHHTEGST	0.271	~ 8.61×10^{-05}	31400	0.1

Table S2: Extinction coefficient of ligands at 335 nm.

Luminescent emission of chelates:

Figure S29 shows the luminescence spectral profile of BHHTEGST-Eu, BHHCT-Eu commercial and BHHCT-Eu, obtained by preparation of chelates (5.0 μM) in Fluorescence Enhancing Buffer (FEB) (see page S33); the spectrum was recorded using the Agilent Cary Eclipse Fluorescence Spectrophotometer. Both chelates displayed a near identical luminescence emission profile, suggesting that the tetraethylene glycol NHS moiety has negligible interaction with the diketone-Eu^{3+} complex.

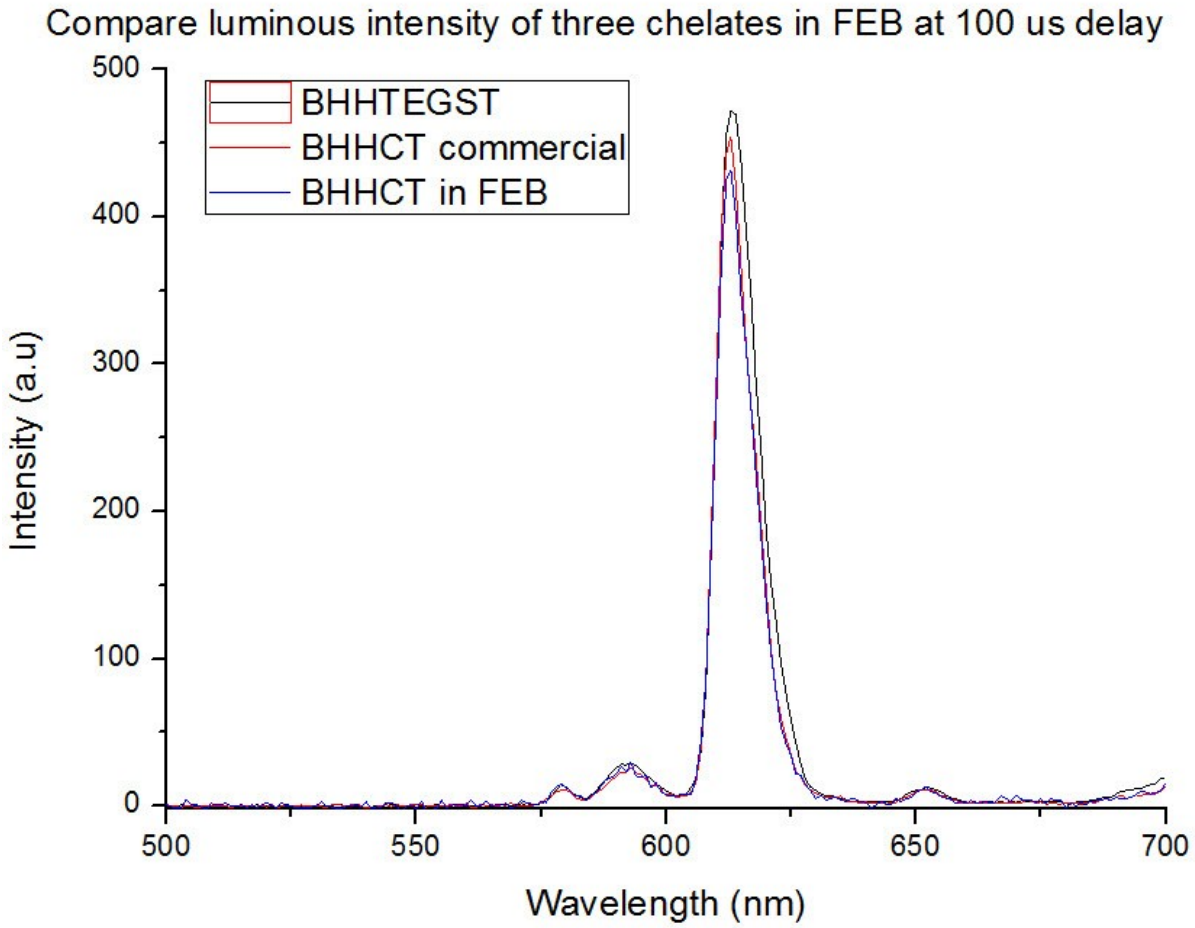


Figure S29: Luminescence emission of BHHCT-Eu, BHHCT-Eu (commercial) and BHHTEGST-Eu (5.0 μM) in 1X FEB solutions. Excited at 335 nm and maximum luminescence emission observed at 613 nm.

Luminescent emission of conjugated antibody with chelate:

Purified antibody conjugates [G2O3-$BHHTEGST_{19}$, G2O3-$BHHTEGST_{14}$ and G2O3-$BHHTEGST_{10}$] (1-2 μM) were added to $NaHCO_3$ buffer (100 mM; pH=8) followed by excess amount of europium chloride (10 eq.) to obtain the luminescent emission of conjugates as

shown in Figure S30. Luminescent emission intensity was observed to be correlated to the number of ligands attached per antibody, increasing ligand count results in higher luminescence intensity. With reference to Figure S31, it can be seen that the luminescence intensity displayed a good linear correlation to the number of ligand attached to antibody.

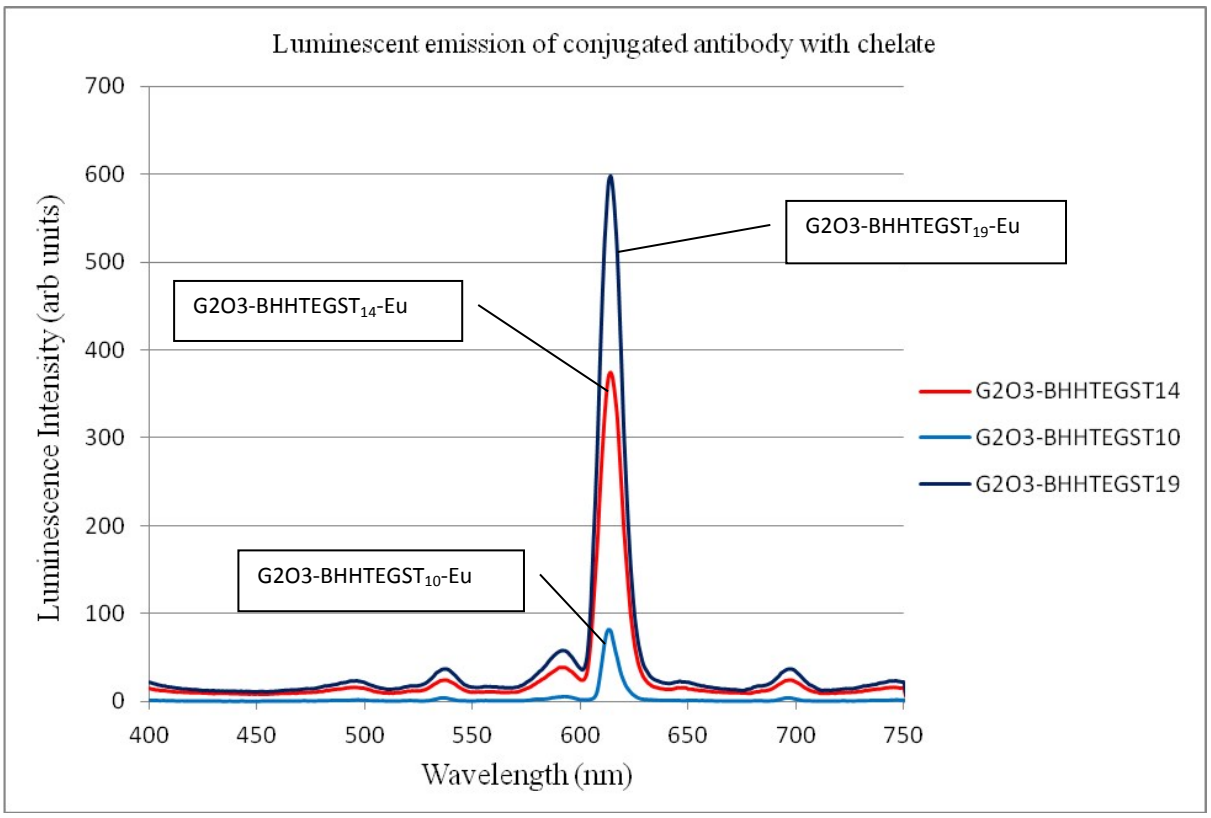


Figure S30: Luminescence profile of conjugated antibodies; [G2O3-BHHTEGST$_{19}$-Eu, G2O3-BHHTEGST$_{14}$-Eu and G2O3-BHHTEGST$_{10}$-Eu]. The small bump at 675 nm is due to Rayleigh scattering arising from excitation at 335 nm.

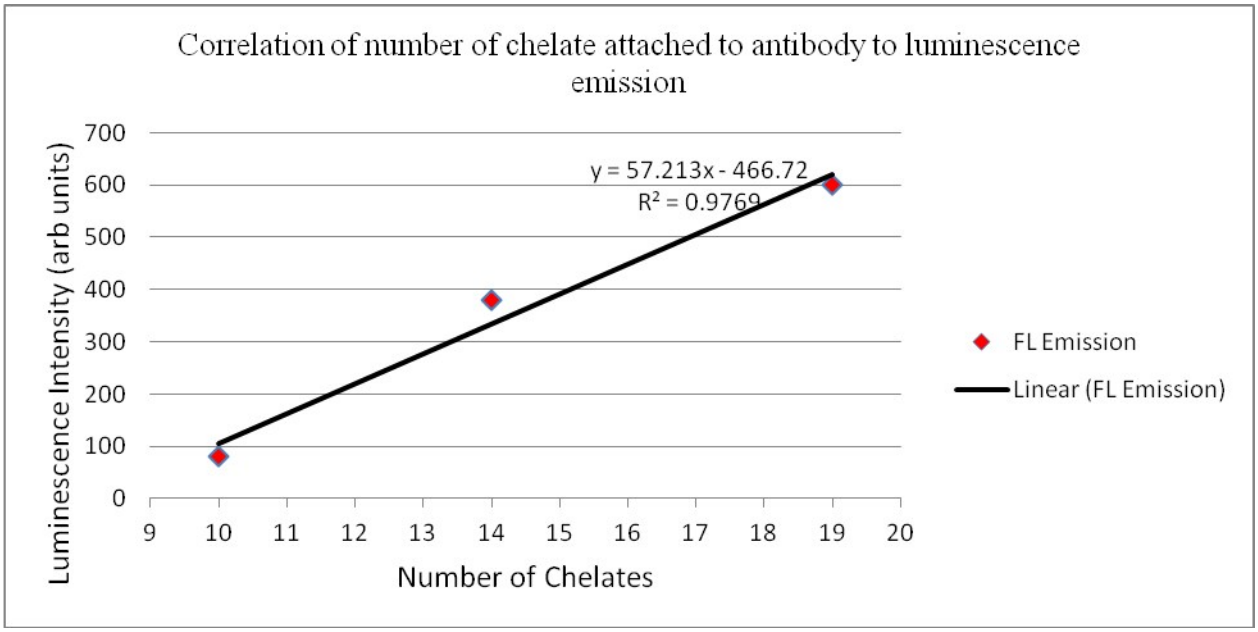


Figure S31: Correlation of number of ligand attached to antibody to luminescence emission of labelled antibody.

Conjugation of BHHCT and BHHTEGST to BSA for stability test:

10mg/ml of Bovine Serum Albumin Fraction V (CAS 9048-46-8 Sigma Aldrich) was prepared in $NaHCO_3$ buffer (100 mM; pH=8). 10 µL of this solution (100 µg) was added to 200 µL of $NaHCO_3$ buffer (100 mM; pH=8) followed by addition of 5 µL of ligands (10 mg/ml in DMF). The reaction mixture was incubated for 1 hr at 37 °C and conjugated BSA was purified on Sephadex G-25 (7 cm length, 0.5 cm ID) using 0.05 M Tris buffer pH=7.8 as eluent. The conjugated fractions were collected and analyzed by using a spectrophotometer (280 nm and 335 nm). [BSA molecular weight 66.5 kDa contains 59 lysine residues with extinction coefficient of 43,824 $M^{-1}cm^{-1}$ at 280 nm.

The ultraviolet absorbance spectrum of conjugated BSA was obtained using a NanoDrop 2000 UV spectrometer (Thermo Scientific) as shown in Figure S32. The number of conjugated ligand per BSA was determined to be BSA-$BHHTEGST_{19}$ and BSA-$BHHCT_{22}$.

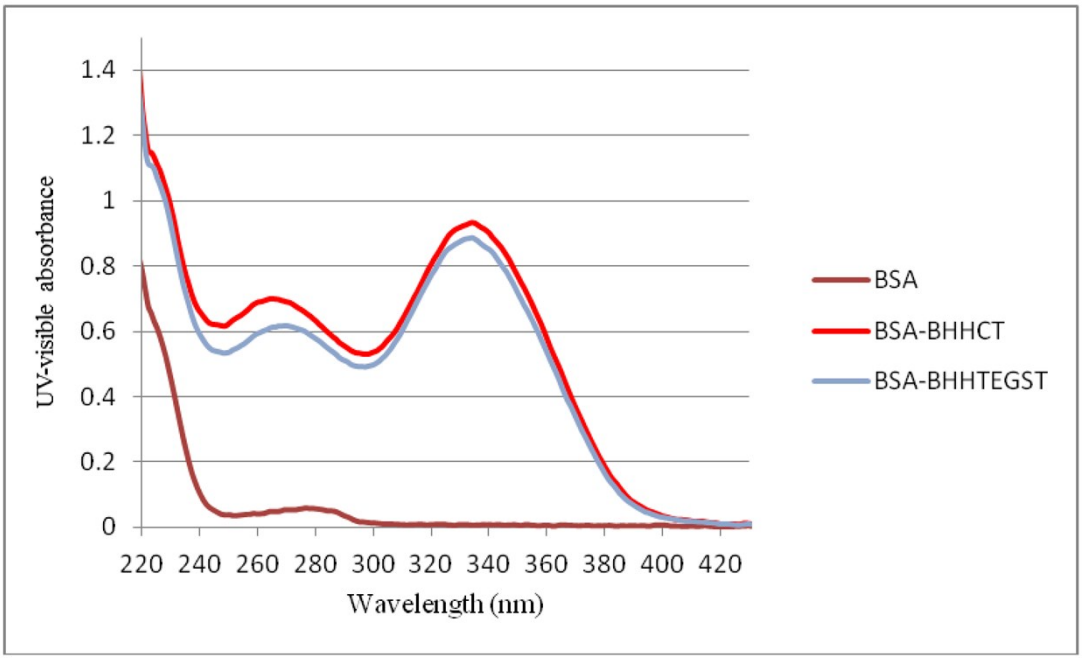


Figure S32: UV-visible profile of BSA and BSA conjugated to BHHTEGST & BHHCT ligands

Stability test of chelates:

Solutions (1 ml each) of the BSA-BHHTEGST$_{19}$-Eu and BSA-BHHCT$_{22}$-Eu with concentration of 1.0 µM in Tris-HCl buffer (0.05 M, pH 7.8) were prepared in a quartz cuvette containing europium chloride (1.5 equivalent of ligand), respectively. The solutions were then combined with the same volume of EDTA (10^{-7} to 10^{-1} M) and after incubation for 5-10 min. the luminescence intensity was observed on the Cary spectrophotometer and plotted against EDTA concentration to generate the graph shown in Figure S33. BSA-BHHTEGS-Eu and BSA-BHHCT-Eu display similar stability in the presence of EDTA; the luminescence intensity of both bioconjugates decrease markedly at concentrations higher than 1.0×10^{-4} M of EDTA, suggesting that EDTA complexes the Eu^{3+} and makes it unavailable to the ligands. The results of the EDTA stability titration with BHHCT are in accordance with the previous study by Zhang, L. *et al*.[7]

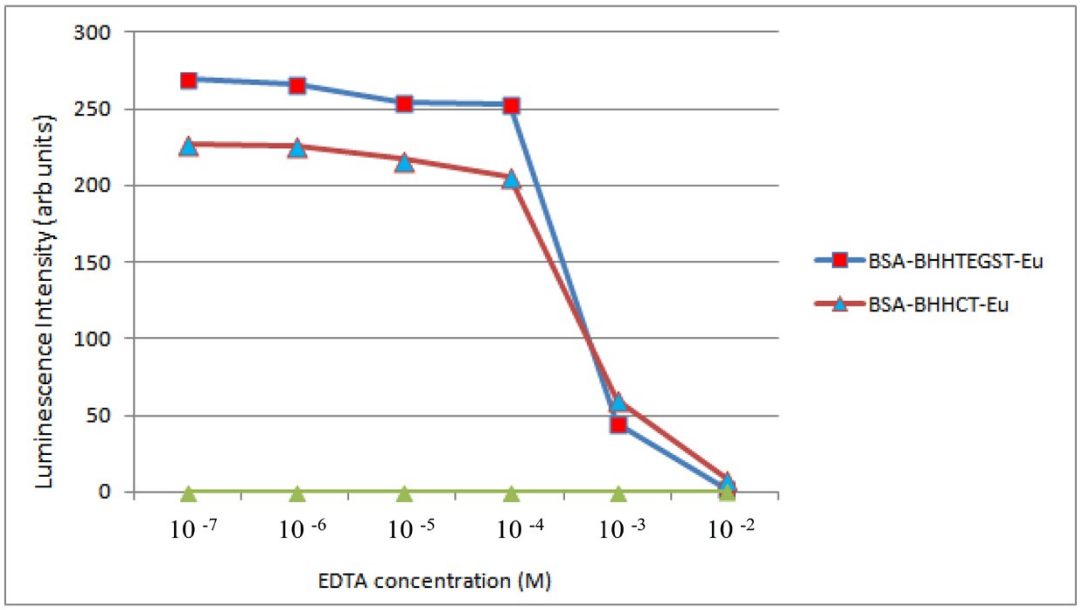


Figure S33: Luminescence intensities of BSA-BHHBTEGST$_{19}$-Eu and BSA-BHHCT$_{22}$-Eu chelates at different concentration of EDTA (10^{-7} – 10^{-2} M).

Solubility and stability of Conjugates:

To obtain maximum luminescence of IgG (G2O3) immunoconjugates, a high ligand/Ab ratio is preferred. Consequently, it is important to prepare a pure and stable immunoconjugate for labelling applications. In our experiments, coupling of BHHCT ligand to antibody (IgG, G2O3) resulted in a cloudy mix of suspended colloids. This has been observed even in diluted solution (0.5-0.1 mg/ml G2O3) which makes it difficult to purify the conjugated antibody. On the other hand BHHTEGST was observed not to suffer from this limitation. To highlight the biocompatibility of our modified europium ligand, the same amount of BHHCT and BHHTEGST (4-5 µL from [10 mg/ml ligand in DMF]; ~50 fold equimolar ligand to antibody) was added to 100 µg antibody (IgG, G2O3) in 100 µL $NaHCO_3$ buffer (100 mM pH 8.5). Following addition of BHHCT to IgG, the reaction mixture was observed to become cloudy with suspended colloids whereas BHHTEGST conjugation with G2O3 results in a clear solution. The reaction mixture for each was diluted with 1 mL buffer and transferred to a quartz cuvette for better visualization. As shown in Figure S34, the conjugation of BHHCT to antibody G2O3 results in a cloudy suspension (A) whilst the BHHTEGST conjugate is a clear solution (B). Europium chloride solution (10 µl, 3 mM) was then added and luminescence excited using a 365 nm UV LED torch G2O3-BHHCT-Eu (C) G2O3-BHHTEGST-Eu (D). To more accurately quantify the presence of the suspension, both conjugates were analysed using a spectrometer set to OD600 mode; BHHCT-G2O3 optical density was reported as 0.646 whereas G2O3- BHTEGST returned a value of zero.

These results support our claim that BHHTEGS conjugate (G2O3-BHHTEGST) possesses superior aqueous stability and biocompatibility when compared to BHHCT conjugate (G2O3-BHHCT).

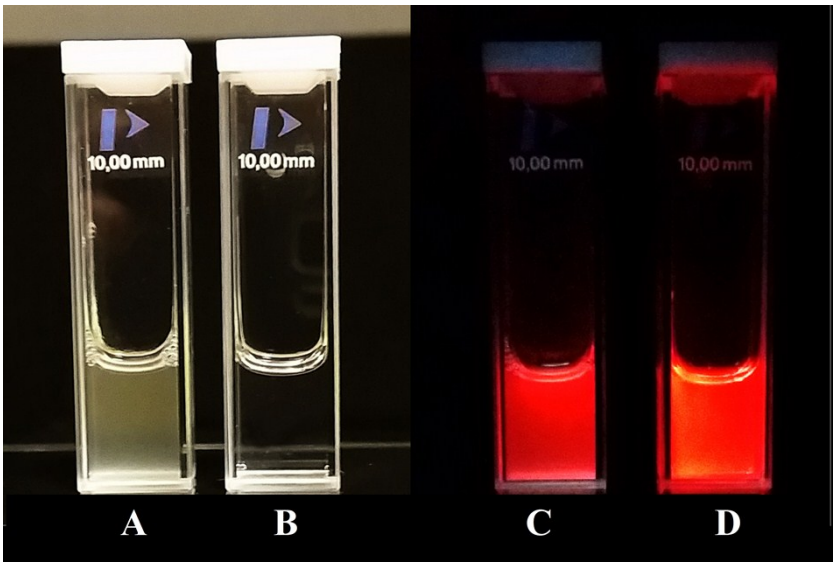


Figure S34: Visualization of cloudy suspended colloids of G2O3-BHHCT (A) and clear solution of G2O3-BHHTEGST (B); after addition of Eu^{3+} A and B visualized under 365 nm UV radiation resulted in C and D, respectively.

Luminescence of chelates in water, deuterium oxide and fluorescence enhancing buffer (FEB) solutions

Preparation of Fluorescence Enhancing Buffer (FEB) 10 X

A 44 mL solution of 0.1M sodium hydroxide solution was prepared and the pH was adjusted to 4.7 with glacial acetic acid, then 1% by volume of Triton X-100 was added. Trioctylphosphine oxide (TOPO; MW 386, 38 mg) was dissolved in ethanol (5 mL) and added to the sodium acetate solution (1.25 mL), 1X FEB was used for the experiments.[8]

Experimental:

1.40 mg HPLC pure lyophilized powder of BHHTEGST and 1.00 mg of BHHCT & BHHCT commercial were accurately weighed (0.01 mg accuracy scale) and dissolved in 500 µL of DMF. Then 10 µL was diluted in 500 µL of [MQ water/ deuterium oxide & FEB] then10 µL of each mixture was added to 1 mL (a) MQ water (b) deuterium oxide (c) 1X FEB (each containing europium chloride 50 µM) in a quartz cuvette for each ligand respectively. Then luminescence intensity was measured using the Cary fluorescence spectrophotometer (Ligand concentration 5.0 µM) as shown in Figure 35 and 36.

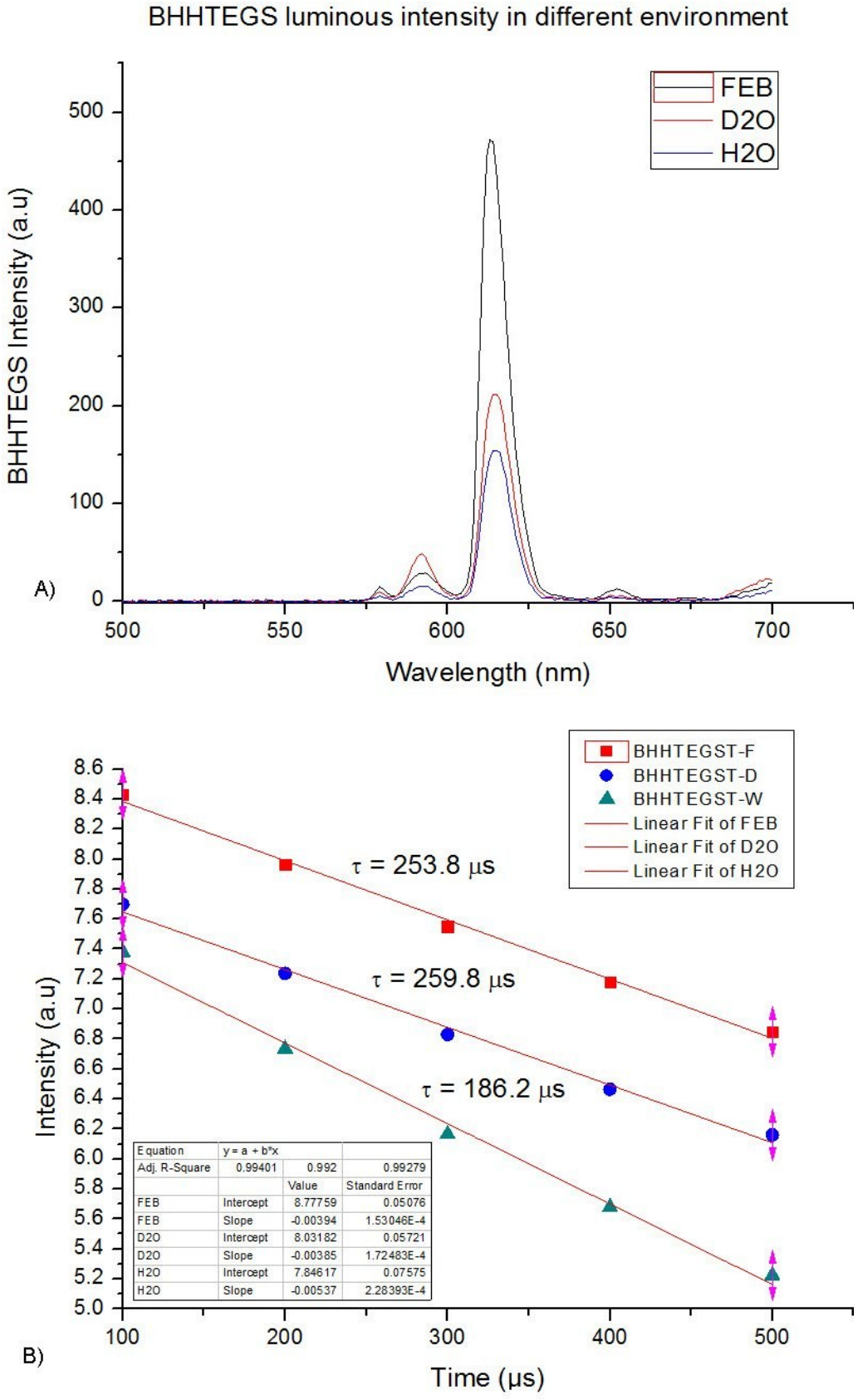
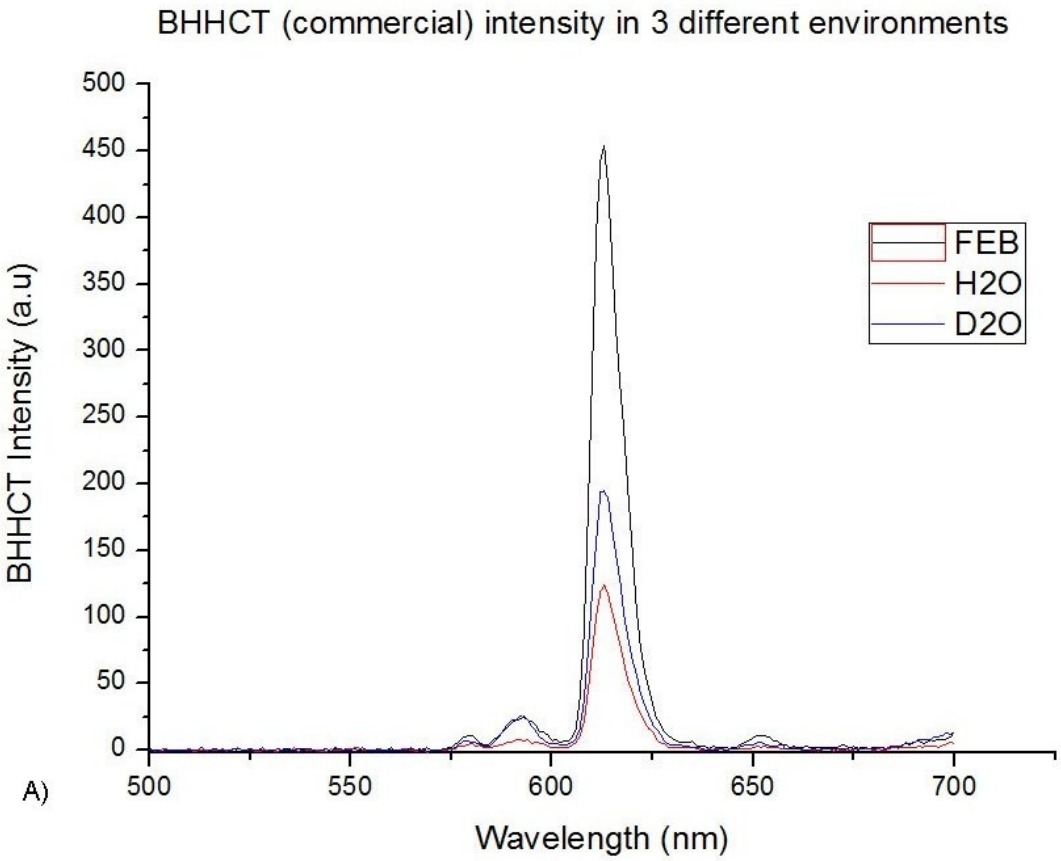
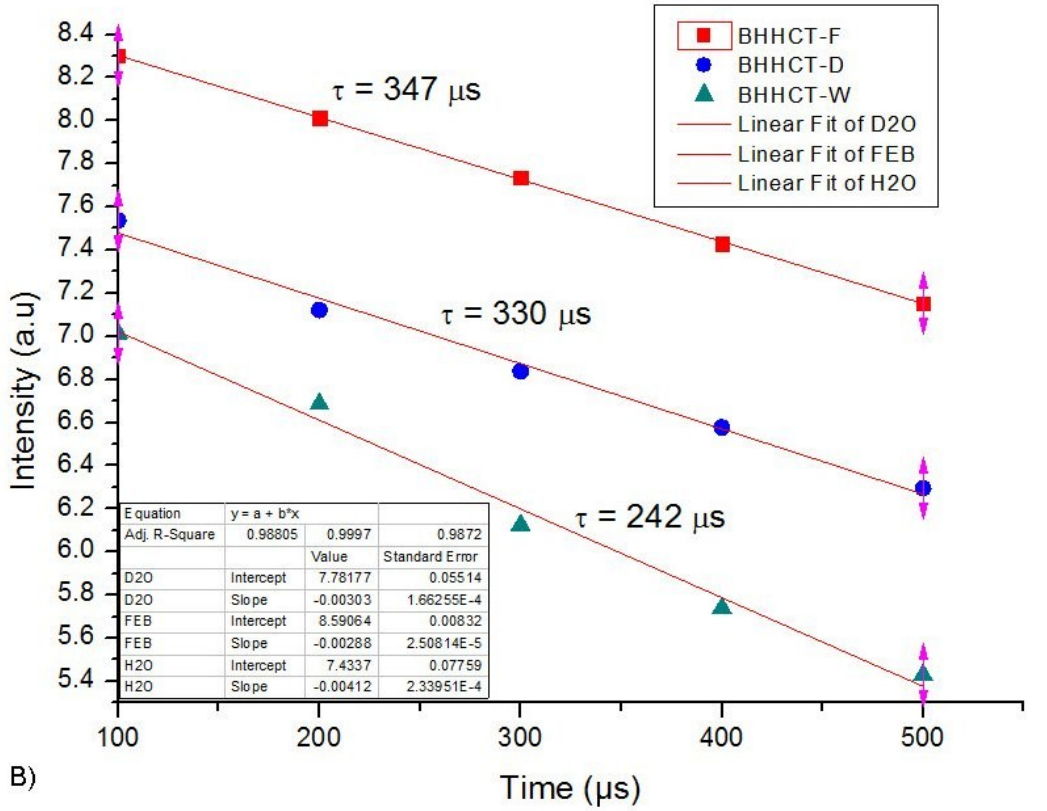


Figure S35A: Luminescence emission of BHHTEGST-Eu (5.0 μM) in water, deuterium oxide and FEB 1X solutions. **S35B:** Luminescence lifetime of BHHCT in FEB, D_2O and water.





Equation	y = a + b*x		
Adj. R-Square	0.98805	0.9997	0.9872
		Value	Standard Error
D2O	Intercept	7.78177	0.05514
D2O	Slope	-0.00303	1.66255E-4
FEB	Intercept	8.59064	0.00832
FEB	Slope	-0.00288	2.50814E-5
H2O	Intercept	7.4337	0.07759
H2O	Slope	-0.00412	2.33951E-4

Figure S36A: Luminescence emission of BHHCT (commercial)-Eu (5.0 μM) in water, deuterium oxide and FEB 1X solutions. **S36B:** Luminescence lifetime of BHHCT in FEB, D_2O and water.

QY DATA

Ligands	ε_{335} nm	Life time µs			Luminescence Emission (in FEB)	QY	Brightness $dm^3\ mol^{-1}\ cm^{-1}$
		FEB	D2O	H2O			
BHHCT Comm	30300	347	330	242	5047	0.25[9]	7575
BHHTEGST	31400	254	260	186	6225	~0.23	7128

Table S3: molar extinction coefficient, Life time and quantum yield for BHHCT and BHHTEGST.

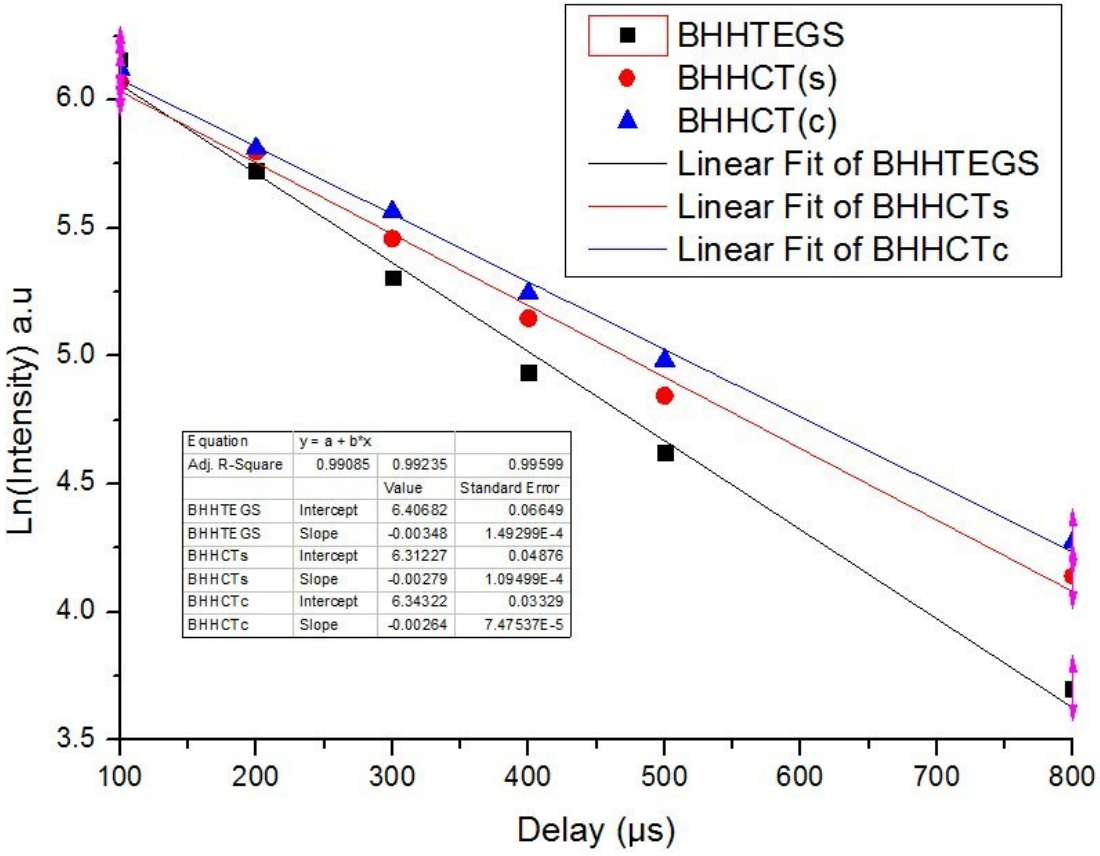


Figure S37: Ln plot of intensity over the period 0 to 800 µs for BHHCT (c) purchased from Sigma, BHHCT(s) synthesized in our labs and BHHTEGST. The 'goodness of fit' R^2 for each linear interpolation was 0.996, 0.992 and 0.99 respectively; the negative reciprocal of the slope is used to calculate the luminescence lifetime value shown in Table S3 and the area under the curve of S38.

Quantum Yield Calculations:

As noted in the text, whilst BHHTEGST is clearly a derivative of BHHCT with the same parent molecule, we observe a small but significant difference in molar extinction coefficient, slightly more intense emission on initial excitation and a shorter luminescence lifetime.

Using these experimentally determined parameters, we used the method reported by Latva *et al.* to determine QY of BHHTEGST.[10]

$$\phi_x = \phi_{ref} \frac{I_{Tx}.\varepsilon_{ref}.C_{ref}}{I_{Tref}.\varepsilon_x.C_x}$$

Where ε represents the molar extinction coefficient at the excitation wavelength, C is the concentration and IT represents the integrated luminescent emission. The subscripts $_{ref}$ and $_x$ refer to the reference compound with known QY and the unknown compound respectively. The concentrations were the same for each compound enabling us to simplify the equation to:

$$\phi_x = .25 \frac{154478 \times 30300}{164389 \times 31400}$$

Which gives us a figure of 0.227 for BHHTEGST quantum yield.

Deslandes *et al.* report a method of evaluating actual efficiency of a luminescent label by taking into account the brightness as determined by the product of the extinction coefficient at the excitation wavelength and the luminescence quantum yield.[11] Using this parameter, we arrive at a figure of 7575 dm^3 mol^{-1} cm^{-1} for BHHCT and 7128 for BHHTEGST. These values are substantially above the lower limit ~300 dm^3 mol^{-1} cm^{-1} reported for the brightness characterizing an efficient luminescent lanthanide complex.[12]

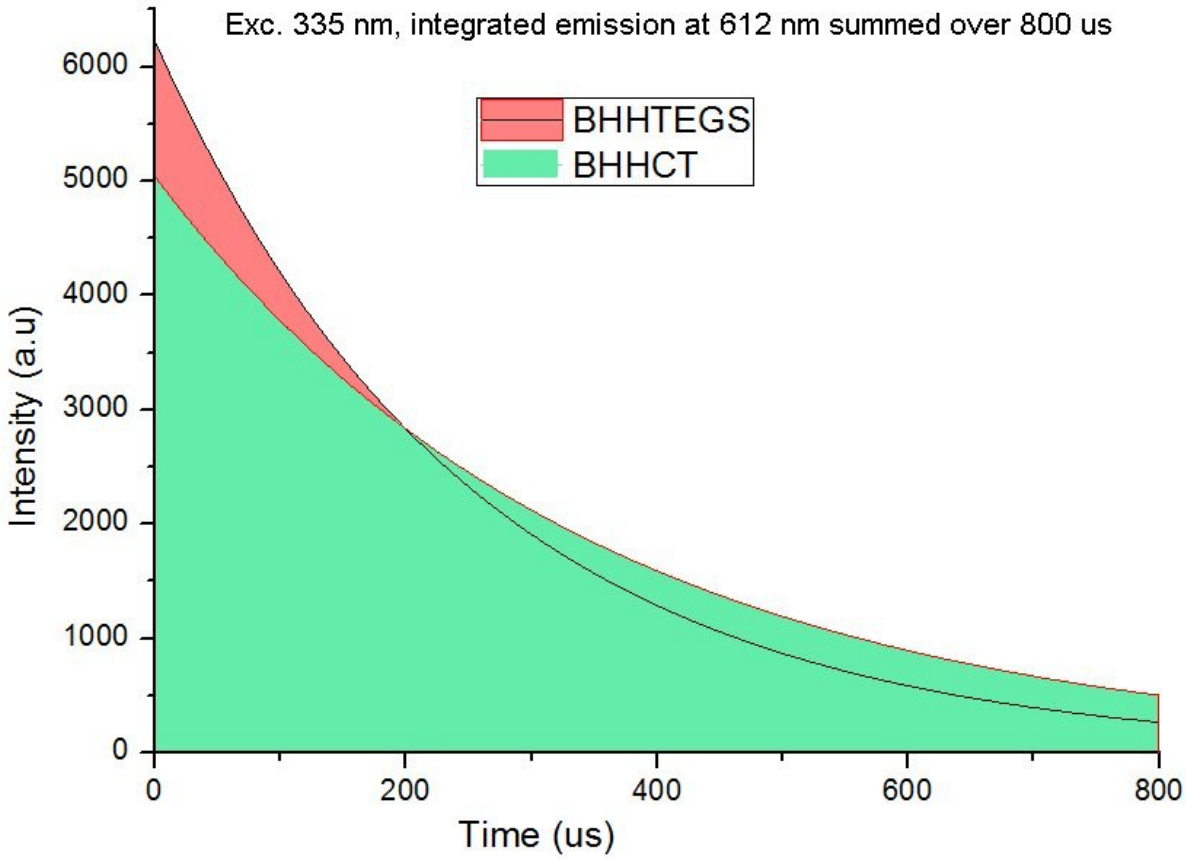


Figure S38: The area under the curve was calculated at 1 nm resolution over the interval 0-800 μs using the exponential function $I_T = 5047\ e^{-T/347}$ for BHHCT and $I_T = 6225\ e^{-T/254}$ for BHHTEGST. The integral for each was then used to calculate quantum yield; the integral for BHHCT was 164,389 and 158,478 for BHHTEGST.

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