Water-solubilisation and bio-conjugation of a red-emitting BODIPY fluorescent marker

Song Lin Niu,[¶a] Cédrik Massif,[¶b,c] Gilles Ulrich,[a] Raymond Ziessel,*[a] Pierre-Yves Renard,[b,c,d] and Anthony Romieu*[b,c]

[a]*S. L. Niu, Dr. G. Ulrich, Dr. R. Ziessel*
LCOSA, ECPM, UMR 7515, CNRS-UdS, 25 rue Becquerel, 67087 Strasbourg Cedex 02, France
Fax (+33)3-68-85-27-61
E-mail: ziessel@unistra.fr

[b]*C. Massif, Dr. A. Romieu, Pr. P.-Y. Renard*
Equipe de Chimie Bio-Organique, COBRA - CNRS UMR 6014 & FR 3038, rue Lucien Tesnière, 76131 Mont-Saint-Aignan, France
Fax (+33)2-35-52-29-59
E-mail: pierre-yves.renard@univ-rouen.fr or anthony.romieu@univ-rouen.fr

[c]*C. Massif, Dr. A. Romieu, Pr. P.-Y. Renard*
Université de Rouen, Place Emile Blondel, 76821 Mont-Saint-Aignan, France

[d]*Pr. P.-Y. Renard*
Institut Universitaire de France, 103 Boulevard Saint-Michel, 75005, Paris, France

[¶]These authors contributed equally to this work.

Supporting Information

Experimental: Detailed synthetic procedures for compounds 2-9.

General

Reversed-phase column flash-chromatographies were performed on octadecyl-functionalised silica gel (mean pore size 60 Å, 37-74 μm) from Aldrich. *N*-Hydroxysulfosuccinimide (sulfo-NHS) was purchased from Pierce. NMP was dried by distillation over BaO. DIEA was distilled from CaH_2 and stored over BaO. Disulfonated linker α-sulfo-β-alaninyl-α-sulfo-β-alanine (diethylammonium salt) and sulfoindocyanine dye Cy 5.0 were prepared using literature procedures.[1,2] Synthesis of BODIPY **3** has been already reported elsewhere.[3] BSA (bovine serum albumin) protein was purchased from Sigma and anti-HA 12CA5 monoclonal antibodies were provided by Dr. Hervé Volland (iBiTecS, Laboratoire d'Etudes et de Recherches en Immuno-analyse, Commissariat à l'Energie Atomique, Gif-sur-Yvette, F-91191, France). The HPLC-gradient grade CH_3CN and CH_3OH were obtained from Fisher Scientific. Buffers ($NaHCO_3$ and PBS) and aq. mobile-phases for HPLC were prepared using water purified with a Milli-Q system (purified to 18.2 MΩ.cm). Triethylammonium acetate (TEAA, 2.0 M) and triethylammonium bicarbonate (TEAB, 1.0 M) buffers were prepared from distilled triethylamine and glacial acetic acid or CO_2 gas.

Instruments and methods

Ion-exchange chromatography (for desalting water-soluble BODIPY) was performed with an Econo-Pac® Disposable chromatography column (Bio-Rad, #732-1010) filled with an aq. solution of Dowex® 50WX8-400 (Alfa Aesar, ~ 7 g for 10 mg of dye, 15 × 50 mm bed), regenerated using aq. 10% HCl solution and equilibrated with deionised water. Size-exclusion chromatography (for purification of fluorescently labelled proteins) was performed with an Econo-Pac® Disposable chromatography column (Bio-Rad, #732-1010) filled with an aq. solution of Sephadex® G-25 Fine (Amersham Biosciences AB, 15 × 40 mm bed), equilibrated with PBS (0.01 M phosphate, 0.015 M NaCl, pH 7.5). ^1H and ^{13}C spectra were recorded on a Bruker DPX 300 spectrometer (Bruker, Wissembourg, France). Chemical shifts are expressed in parts per million (ppm) from DMSO-d_6 (δ_H = 2.54).[4] *J* values are expressed in Hz. Analytical HPLC was performed on a Thermo Scientific Surveyor Plus instrument equipped with a PDA detector. Semi-preparative HPLC was performed on a Thermo Scientific SPECTRASYSTEM liquid chromatography system (P4000) equipped with a UV-visible 2000 detector. Mass spectra were obtained with a Finnigan LCQ Advantage MAX (ion trap) apparatus equipped with an electrospray source. UV-visible absorption spectra were obtained on a Varian Cary 50 scan spectrophotometer. Fluorescence spectroscopic studies were performed with a Varian Cary Eclipse spectrophotometer. The absorption spectra of water-soluble BODIPY **9** and the corresponding fluorescent bio-conjugates were recorded (220-850 nm) in PBS (0.1 M phosphate + 0.15 M NaCl, pH 7.4 for **9** and 10 mM phosphate + 15 mM NaCl, pH 7.4 for bio-conjugates) (concentration: 1.0-10.0 μM) at 25 °C. Emission spectra were recorded under the same conditions after excitation at 600 nm (excitation and emission filters : auto, excitation and emission slit = 5 nm) in PBS. Relative quantum yields were measured in PBS at 25 °C by a relative method using sulfoindocyanine dye Cy 5.0 (Φ_F =

[1] A. Romieu, D. Brossard, M. Hamon, H. Outaabout, C. Portal and P.-Y. Renard, *Bioconjugate Chem.*, 2008, **19**, 279.
[2] R. B. Mujumdar, L. A. Ernst, S. R. Mujumdar, C. J. Lewis and A. S. Waggoner, *Bioconjugate Chem.*, 1993, **4**, 105.
[3] T. N. Singh-Rachford, A. Haefele, R. Ziessel and F. N. Castellano, *J. Am. Chem. Soc.*, 2008, **130**, 16164.
[4] H. E. Gottlieb, V. Kotlyar and A. Nudelman, *J. Org. Chem.*, 1997, **62**, 7512.

0.20 in PBS)[2] as a standard. The following equation was used to determine the relative fluorescence quantum yield:

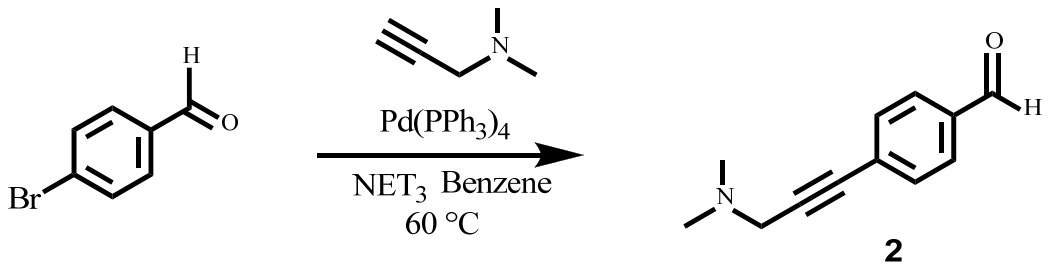
$$\Phi_F(x) = (A_S/A_X)(F_X/F_S)(n_X/n_S)^2\Phi_F(s)$$

Where A is the absorbance (in the range 0.01-0.1 A.U.), F is the area under the emission curve, n is the refractive index of the solvents (at 25 °C) used in measurements (n = 1.337 for PBS), and the subscripts s and x represent standard and unknown, respectively.

High-performance liquid chromatography separations

Three chromatographic systems were used for the analytical experiments and the purification steps. **System A:** RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 4.6 × 150 mm) with CH_3OH and 0.1% aq. trifluoroacetic acid (aq. TFA, 0.1%, v/v, pH 2.2) as eluents [20% CH_3CN (5 min), followed by linear gradient from 0 to 100% (32 min) of CH_3OH] at a flow rate of 1.0 mL min^{-1}. Triple UV-vis detection was achieved at 254, 530 and 640 nm. **System B:** RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 μm, 4.6 × 100 mm) with CH_3CN and aq. TFA, 0.1% as eluents [20% CH_3CN (5 min), followed by linear gradient from 0 to 100% (32 min) of CH_3CN] at a flow rate of 1.0 mL min^{-1}. Triple UV-vis detection was achieved at 254, 530 and 640 nm. **System C:** system A with CH_3CN and aq. triethylammonium acetate (TEAA, 100 mM, pH 7.0) as eluents. **System D:** semi-preparative RP-HPLC (Varian Kromasil C_{18} column, 10 μm, 21.2 × 250 mm) with CH_3CN and and aq. triethylammonium bicarbonate (TEAB 50 mM, pH 7.5) as eluents [5% CH_3CN (10 min), followed by linear gradient from 5 to 100% (47 min) of CH_3CN] at a flow rate of 16.0 mL min^{-1}. Visible detection was achieved at 590 nm.

3-(Dimethylamino)-1-(4-formylphenyl)propyne (2).[5]

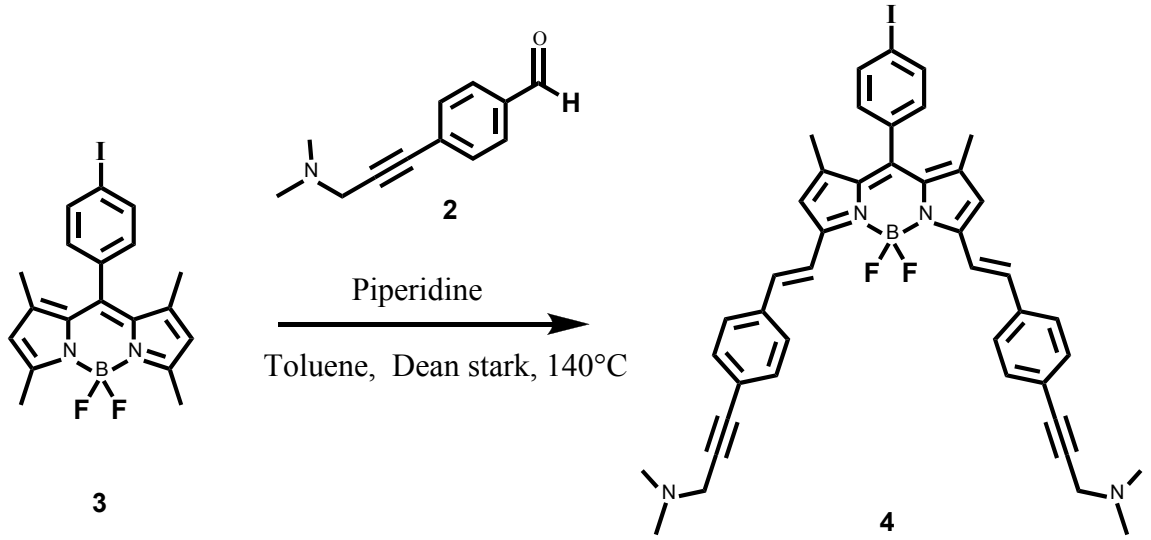


2

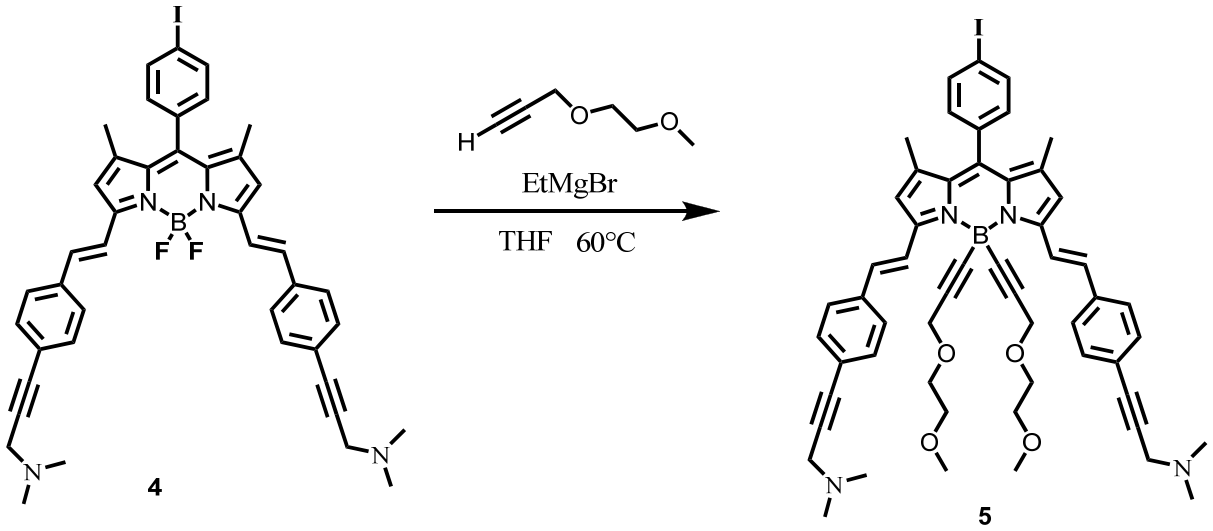
To a degassed solution of 4-bromobenzaldehyde (1.0 g, 5.40 mmol) in benzene (3 mL) and TEA (3 mL), were added [$Pd(PPh_3)_4$] (0.36 g, 0.324 mmol) and 1-dimethylamino-2-propyne (0.67 g, 8.10 mmol) under argon. The mixture was stirred at 60 °C for 10 h until the complete consumption of the starting material was observed by TLC. The mixture was then evaporated, and a chromatography on silica gel (CH_2Cl_2 100%, and then CH_2Cl_2-CH_3OH, 97 : 3, v/v) afforded **2** (1.13 g, quantitative yield). δ_H(200MHz, $CDCl_3$) 10.00 (s, 1H), 7.82 (d, J = 8.3 Hz, 2H), 7.58 (d, J = 8.3 Hz, 2H), 3.54(s, 2H), 2.42 (s, 6H); MS (FAB+, *m*-NBA as a matrix): *m/z* 188.1 $[M + H]^+$, calcd for $C_{12}H_{13}NO$ 187.09; Found C, 76.64; H, 6.82 ; N, 7.17. $C_{12}H_{13}NO$ requires C, 76.97; H, 6.99; N, 7.48%.

[5] M. Lemhadri, H. Doucet and M. Santelli, *Synthesis*, 2005, 1359.

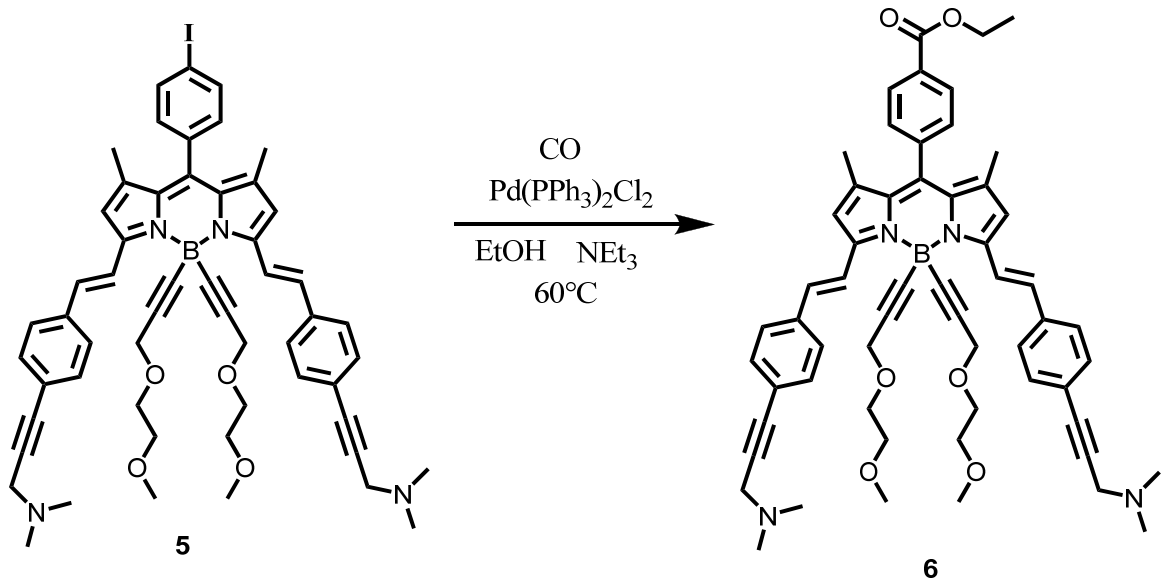
Preparation of the BODIPY dye (8) according to Scheme 1.



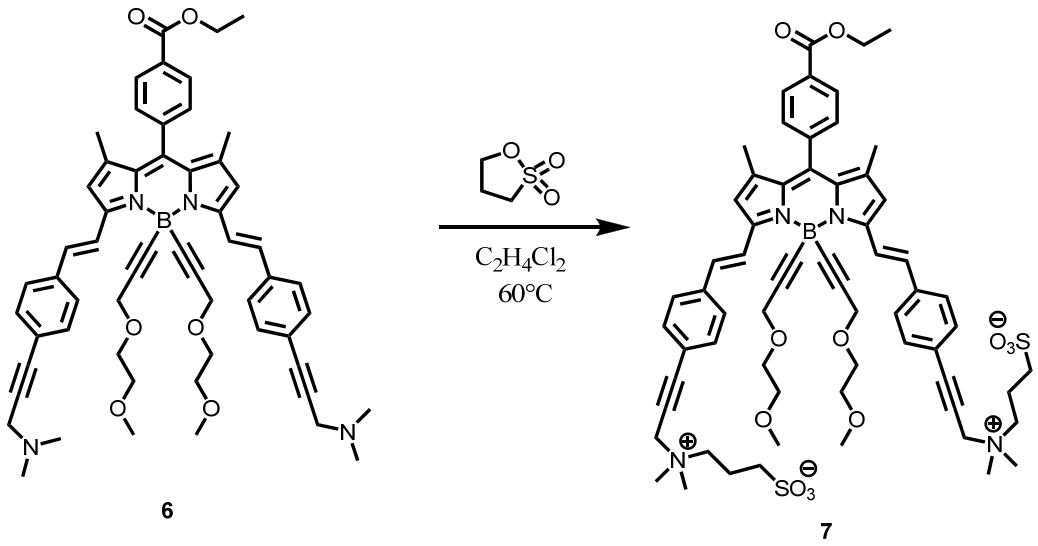
A mixture of 4, 4-difluoro-1, 3, 5, 7-tetramethyl-8-(4'-iodo-phenyl)-4-bora-3a, 4a-diaza-s-indacene **3** (500 mg, 1.11 mmol) and 3-(dimethylamino)-1-(4-formylphenyl)propyne **2** (415 mg, 2.22 mmol) in toluene (20 ml) and piperidine (0.5 mL) was heated at 140 °C in a Dean-Stark apparatus for 12 h. Thereafter, the reaction mixture was evaporated, then treated with saturated aq. $NaHCO_3$ solution and deionised water, extracted with CH_2Cl_2. The organic layer was dried with $MgSO_4$ then evaporated to dryness. The resulting crude product was purified by column chromatography on silica gel (CH_2Cl_2-CH_3OH, 97: 3 to 93: 7, v/v) afforded the bis-styryl compound **4** (193 mg, 22%), along with the mono-styryl compound (135 mg, 20%). δ_H(300 MHz, $CDCl_3$) 7.86 (d, J = 7.7 Hz, 2H), 7.71 (d, J = 16.3 Hz, 2H), 7.56 (d, J = 7.9 Hz, 4H), 7.46 (d, J = 7.8 Hz, 4H), 7.22 (d, J = 16.4 Hz, 2H), 7.08 (d, J = 7.7 Hz, 2H), 6.65 (s, 2H), 3.52 (s, 4H), 2.40 (s, 12H), 1.48 (s, 6H); δ_C(75 MHz, $CDCl_3$) 152.9, 142.2, 138.6, 136.4, 135.9, 134.8, 133.5, 130.6, 127.6, 123.8, 119.9, 118.4, 95.1, 86.7, 85.7, 48.9, 44.5, 15.2; MS (FAB+, *m*-NBA as a matrix): *m/z* 788.2 $[M + H]^+$, calcd for $C_{43}H_{40}BF_2IN_4$ 787.24; Found; C, 65.22; H, 4.64; N, 6.82. $C_{43}H_{40}BF_2IN_4$ requires C, 65.50; H, 5.11; N, 7.11%; $\lambda_{max}(CH_2Cl_2)$/nm 646 (ε/dm^3 mol^{-1} cm^{-1} 110 600); λ_{max} em (CH_2Cl_2)/nm 661 (Φ_F 0.46).



To a solution of 3-(2-methoxyethoxy)-1-propyne (68 mg, 0.60 mmol) in dry THF (5 mL) under argon in a schlenk flask, was added 1.0 M EtMgBr in THF (0.52 mL), and the solution was stirred at 60 °C for one hour. The resulting anion was then transferred *via* cannula to the solution of **4** (120mg, 0.15 mmol) dissolved in a separate schlenk flask in dry THF (3 mL) under argon. The mixture was stirred at 60 °C for 15 min, until complete consumption of the starting material was observed by TLC, then H_2O (3 mL) was added. The mixture was then washed with water, brine then extracted with CH_2Cl_2. The organic layer was dried on $MgSO_4$ then evaporated. The crude product was purified by column chromatography on silica gel (CH_2Cl_2-CH_3OH, 93 : 7, v/v) afforded the compound **5** (104 mg, 70%). δ_H(300 MHz, $CDCl_3$): δ 8.21 (d, J = 16.5 Hz, 2H), 7.86 (d, J = 7.7 Hz, 2H), 7.58 (d, J = 8.0 Hz, 4H), 7.48 (d, J = 8.1 Hz, 4H), 7.13 (m, 4H), 6.66 (s, 2H), 4.15 (s, 4H), 3.56 (s, 4H), 3.49 (m, 4H), 3.20 (s, 6H), 3.16 (m, 4H), 2.43 (s, 12H), 1.47 (s, 6H); δ_C(75 MHz, $CDCl_3$) 152.0, 140.7, 138.5, 138.0, 137.0, 135.1, 133.8, 132.5, 130.7, 127.4, 123.4, 121.8, 118.7, 92.1, 86.0, 77.6, 77.2, 76.8, 71.7, 68.5, 59.0, 48.7, 44.2, 29.9, 15.4; MS (FAB+, *m*-NBA as a matrix) *m/z* 977.2 $[M + H]^+$; calcd for $C_{55}H_{58}BIN_4O_4$ 975.36; Found; C, 66.12; H, 5.74; N, 5.44. $C_{55}H_{58}BIN_4O_4 \cdot H_2O$ requires C, 66.40; H, 6.08; N, 5.63%; $\lambda_{max}(CH_2Cl_2)$/nm 647 (ε/dm^3 mol^{-1} cm^{-1} 108 500); λ_{max} em (CH_2Cl_2)/nm 659 (Φ_F 0.47).

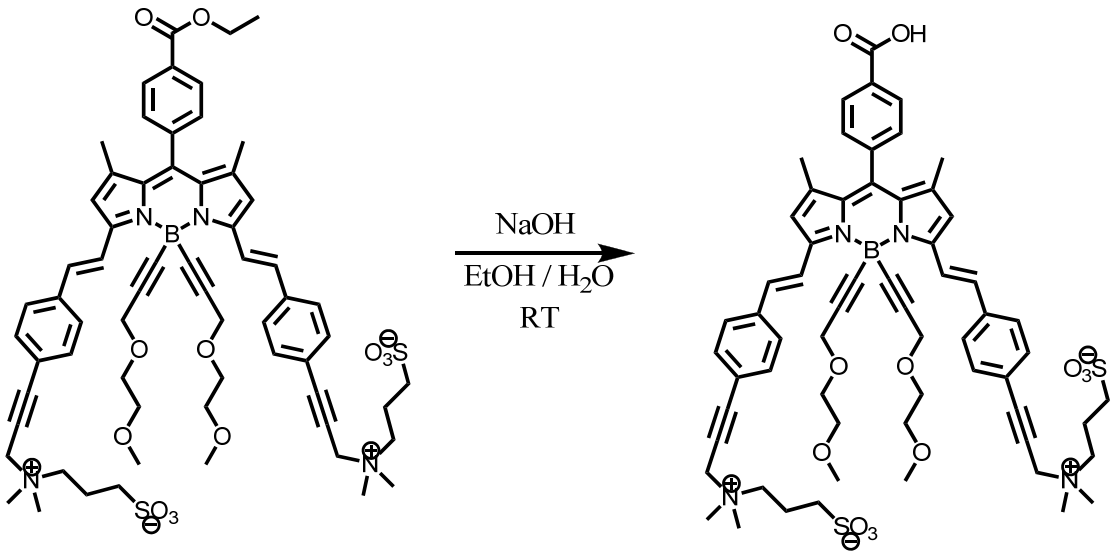


To stirred solution of compound **5** (104 mg, 0.105 mmol) in absolute EtOH (4 mL) and NEt_3 (3 mL) were added $Pd(PPh_3)_2Cl_2$ (8 mg, 0.01 mmol) and then CO gas was bubbled at 60 °C for 5 h. After the solvent was removed, the crude was purified by column chromatography on silica gel (CH_2Cl_2-CH_3OH, 93: 7, v/v), gave the compound **6** (116 mg, quantitative yield). δ_H(300 MHz, $CDCl_3$) 8.24 (d, J = 16.3 Hz, 2H) 8.19 (d, J = 8.5 Hz, 2H), 7.58 (d, J = 8,3 Hz, 4H), 7.48 (d, J = 8.3 Hz, 4H), 7.46 (d, J = 7.9 Hz, 2H), 7.13 (d, J = 16.5 Hz, 2H), 6.65 (s, 2H), 4.43 (q, J = 7.2 Hz, 2H), 4.15 (s, 4H), 3.59 (s, 4H), 3.49 (m, 4H), 3.20 (s, 6H), 3.16 (m, 4H), 2.46 (s, 12H), 1.45 (t, J = 7.3 Hz, 3H), 1.41 (s, 6H); δ_C(75 MHz, $CDCl_3$) 166.2, 152.1, 140.7, 140.3, 138.3, 132.5, 131.3, 130.4, 129.1, 127.4, 123.2, 121.9, 118.8, 92.1, 86.4, 85.4, 71.7, 68.5, 61.6, 59.6, 59.0, 48.7, 46.0, 44.1, 29.9, 15.2, 14.5, 8.8; MS (FAB+, *m*-NBA as a matrix): *m/z* 922.4 $[M + H]^+$, calcd for $C_{58}H_{63}BN_4O_6$ 921.48; Found: C, 73.78, H, 6.70, N, 5.66. $C_{58}H_{63}BN_4O_6 \cdot H_2O$ requires C, 74.03; H, 6.96; N, 5.95%.



6 **7**

To a solution of **6** (50 mg, 540 µmol) in dry 1,2-dichloroethane (3 ml) under argon, was added the 1,3-propanesultone (66 mg, 542 µmol), then the resulting reaction mixture was stirred at 60 °C overnight, until the complete consumption of the starting material was observed by TLC (EtOH-H_2O, 8 : 2, v/v). The precipitate formed was then centrifugalized and washed with 1,2-dichloroethane. The crude product was roughly purified by column chromatography on silica gel (EtOH-H_2O, 7 : 3, v/v) and then recrystallised from CH_3OH-AcOEt to afford the desired compound **7** (36 mg, 60%). It was used in the next step without further purification but interpretable NMR spectra were obtained. δ_H(300 MHz, $CD_3OD/CDCl_3$) 8.24 (d, J = 16.4 Hz, 2H), 8.16 (d, J = 8.1 Hz, 2H), 7.61 (d, J = 8.2 Hz, 4H), 7.55 (d, J = 8.2 Hz, 4H), 7.44 (d, J = 8.3 Hz, 2H), 7.14 (d, J = 16.2 Hz, 2H), 6.67 (s, 2H), 4.49 (s, 4H), 4.39 (q, J = 7.3 Hz, 2H), 4.09 (s, 4H), 3.69 (m, 4H), 3.28 (m, 4H), 3.21 (s, 12H), 3.14 (m, 10H), 2.90 (m, 4H), 2.24 (m, 4H),1.39 (m, 9H); δ_C(75 MHz, $CD_3OD/CDCl_3$) 166.2, 151.5, 140.8, 139.8, 138.5, 132.9, 132.6, 132.4, 131.5, 130.9, 130.1, 128.7, 127.2, 122.5, 119.9, 118.6, 91.8, 91.6,71.1, 67.9, 63.1, 61.4, 59.1, 58.2, 55.3, 41.9, 18.7, 14.7,13.9.



7 **8**

To a solution of compound **7** (36 mg, 0.03 mmol), in EtOH (2 mL) and H_2O (1 mL) was added NaOH (12 mg, 0.3 mmol). The mixture was stirred at room temperature overnight, until complete consumption of the staring material was observed by TLC (EtOH-H_2O, 7: 3, v/v). To the solution was added 2% aq. HCl until neutral, then addition of AcOEt lead to precipitation of the compound. The crude product was recrystallised twice from CH_3OH/AcOEt to give the compound **8** (30 mg, 88%). δ_H(200 MHz, $CD_3OD/CDCl_3$) 8.35-8.14 (m, 4H), 7.65 (m, 8H), 7.40-7.24 (m, 4H), 6.79(s, 2H), 4.63(s, 4H), 4.11(s, 4H), 3.72(m, 4H), 3.48 (m, 4H), 3.28(m, 12H), 3.15(m, 10H), 2.90(m, 4H), 2.30(m, 4H),1.43(m, 6H); δ_C(50 MHz, $CD_3OD/CDCl_3$) 152.8, 142.2, 140.0, 133.8, 128.3, 124.8, 92.8, 92.6, 72.4, 70.3, 69.1, 59.9, 59.0, 56.3, 30.3, 26.4, 20.0, 15.2; MS (FAB+, *m*-NBA as a matrix): *m/z* 1161.6 $[M + Na]^+$; MS (ESI+): *m/z* 1139.13 $[M + H]^+$, calcd for $C_{62}H_{71}BN_4O_{12}S_2$ 1137.46; HPLC (system A): t_R = 28.0 min, purity 89% (max plot 220-700 nm) and 97% (640 nm); λ_{max}(recorded during the HPLC analysis)/nm 366, 592 and 639.

Water-soluble red-emitting BODIPY (9).

(a) Preparation of N-Hydroxysuccinimidyl ester: BODIPY carboxylic acid **8** (6.2 mg, 4.5 μmol) was dissolved in dry NMP (200 μL). 350 μL of a solution of TSTU reagent in dry NMP (10.0 mg, 33.2 μmol) and 9 μL of a 2.0 M solution of DIEA in dry NMP (18 μmol) were added and the resulting reaction mixture was protected from light and stirred at room temperature for 1 h. The reaction was checked for completion by RP-HPLC (system B) and ESI-MS. The resulting *N*-hydroxysuccinimidyl ester was used in the next coupling step without purification. HPLC (system B): t_R = 16.3 min (compared to t_R = 15.1 min for BODIPY carboxylic acid **8**); MS (ESI+): *m/z* 1236.47 $[M + H]^+$, calcd for $C_{66}H_{74}BN_5O_{14}S_2$ 1234.48.

(b) Coupling reaction : α-Sulfo-β-alaninyl-α-sulfo-β-alanine (22 mg, 40.7 μmol) was dissolved in 0.24 M aq. $NaHCO_3$ buffer (pH 8.2, 500 μL) and the resulting solution was cooled to 4 °C. The crude solution of *N*-hydroxysuccinimidyl ester in NMP was added dropwise to this stirred solution. The resulting reaction mixture was left at 4°C overnight. The reaction was checked for completion by RP-HPLC. (system C). Finally, the reaction mixture was quenched by dilution with aq. TEAB (50 mM, pH 7.5) and purified by RP-HPLC (system D, 1 injection, t_R = 22.7-25.0 min). The product-containing fractions were lyophilised to give the TEA salt of water-soluble BODIPY **9**. Desalting by ion-exchange chromatography (followed by lyophilisation) afforded the acid form of **9** as a blue amorphous powder (3.0 mg, yield 47%, mixture of two racemic diastereomers). δ_H(300 MHz; DMSO-d_6) 8.17 (2 H, d, *J* 16.3, -C*H*=CH-BODIPY), 7.97 (2 H, m, Ph-BODIPY), 7.70 (10 H, m, Ph-BODIPY), 7.55 (m, 4 H, -CH=C*H*-BODIPY and Ph-BODIPY), 7.02 (2 H, s, pyrrole-BODIPY), 4.64 (4 H, s, 2 × N-CH_2-C≡C-), 4.05 (4 H, s, 2 × O-CH_2-C≡C-), 3.79-3.37 (14 H, m, 2 × CH_2-C*H*(SO_3H)-CO-, 2 × N-CH_2-$(CH_2)_2$-SO_3^- and 2 × O-CH_2-CH_2-O), 3.15 (12 H, s, 2 × -N(CH_3)$_2$-$(CH_2)_2$-SO_3^-), 3.12 (4 H, m, 2 × O-CH_2-CH_2-O), 3.06 (6 H, s, 2 × OCH_3), 2.54 (4 H, m, 2 × N-$(CH_2)_2$-CH_2-SO_3^-, partially masked by DMSO peak), 2.05 (4 H, m, 2 × N-CH_2-CH_2-CH_2-SO_3^-), 1.41 (6 H, s, 2 × CH_3, BODIPY); (ESI+): *m/z* 1442.07 $[M + H]^+$, MS (ESI-): *m/z* 1439.40 $[M - H]^-$, calcd for $C_{68}H_{81}BN_6O_{20}S_4$ 1439.45; HPLC (system C): t_R = 9.7 min, purity 92%; λ_{max}(PBS)/nm 368 (ε/dm^3 mol^{-1} cm^{-1} 76 860), 642 (ε/dm^3 mol^{-1} cm^{-1} 55 080).

Fluorescent labelling of proteins.

*(a) Conversion of water-soluble BODIPY **9** into amine-reactive derivative* : Water-soluble BODIPY dye carboxylic acid (0.54 mg, 0.37 μmol, weighed in a 0.5 mL Eppendorf tube) was dissolved in deionised water (50 μL). 30 μL of an aq. solution of water-soluble carbodiimide (EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, 1.06 mg, 5.55 μmol)

and 10 µL of an aq. solution of sulfo-NHS (0.17 mg, 0.78 µmol) were sequentially added and the resulting reaction mixture was protected from light and periodically vortexed. The reaction was checked for completion by RP-HPLC (system C). The resulting *N*-hydroxysulfosuccinimidyl ester was used in the next labelling step without purification. HPLC (system C): t_R = 10.3 min (compared to t_R = 9.7 min for water-soluble BODIPY carboxylic acid **9**).

(b) Labelling of antibodies : 45 µL of the solution of *N*-hydroxysulfosuccinimidyl ester (*vide supra*, 185 nmol, 31-fold excess) was added to a 500 µL solution of anti-HA antibodies (1.8 mg/mL, 6 nmol) in phosphate buffer (pH 7.4). The resulting mixture was protected from the light and periodically vortexed. The reaction was left at 4 °C overnight. Thereafter, BODIPY-mAb conjugate was purified by size-exclusion chromatography (*vide supra*). The number of BODIPY per antibody (molar ratio, F/P) was determined spectrophotometrically by measuring their absorbance at 280 and 642 nm and inserting the measured values into the following equation :

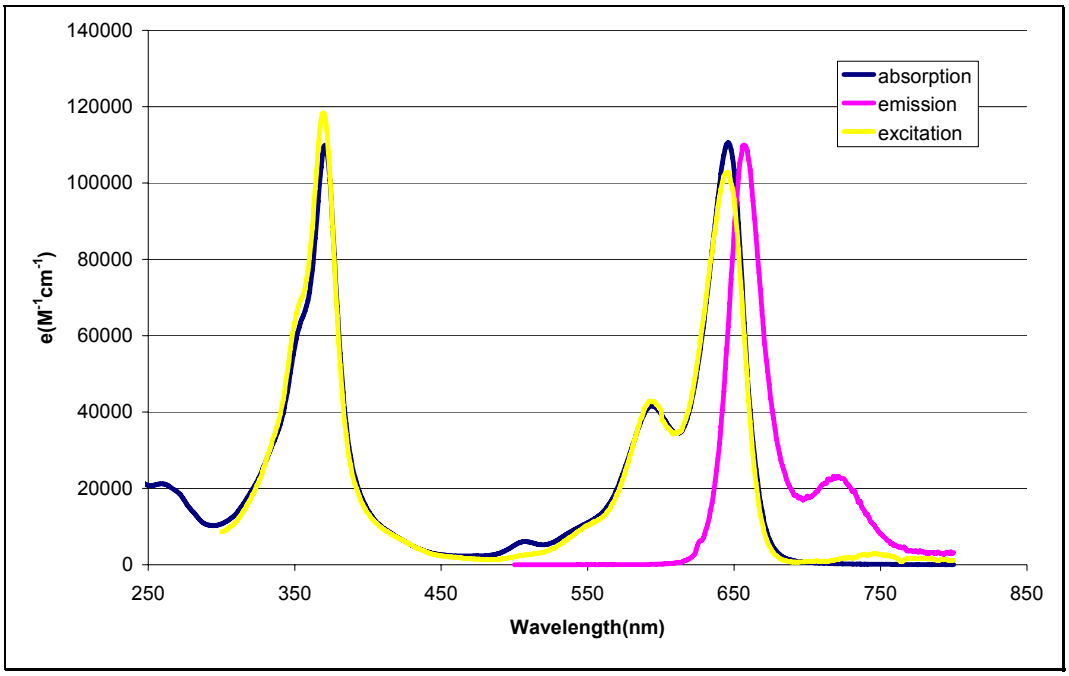
$$F/P = A_{max}\ {}^{P}\varepsilon_{280} / (A_{280}\ {}^{F}\varepsilon_{max} + A_{max}\ {}^{F}\varepsilon_{280})$$

Where A_{280} is the absorbance of the protein at 280 nm, ${}^{P}\varepsilon_{280}$ is the extinction coefficient of the protein at 280 nm, A_{max} is the absorbance of the BODIPY label as its absoprtion maximum, ${}^{F}\varepsilon_{max}$ is the extinction coefficient of the fluorophore at the absorption maximum, and ${}^{F}\varepsilon_{280}$ is the extinction coefficient of the fluorophore at 280 nm. Anti-HA antibodies have an extinction coefficient at 280 nm of 2.95×10^5 dm^3 mol^{-1} cm^{-1}.

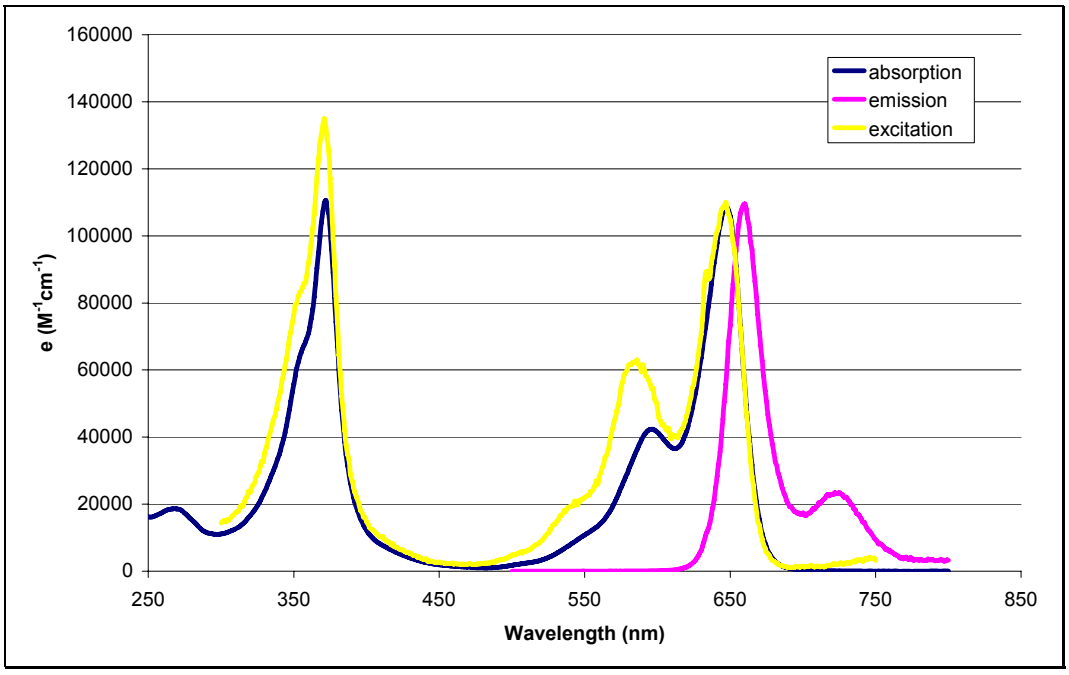
(c) Labelling of BSA : 45 µL of the solution of *N*-hydroxysulfosuccinimidyl ester (*vide supra*, 185 nmol, 13-fold excess) was added to a 500 µL solution of BSA (1.8 mg/mL, 13 nmol) in phosphate buffer (pH 7.4). The resulting mixture was protected from the light and periodically vortexed. The reaction was left at 4 °C overnight. Thereafter, BODIPY-BSA conjugate was purified by size-exclusion chromatography (*vide supra*). The BODIPY per protein ratio (F/P) was determined spectrophotometrically by measuring their absorbance at 280 and 642 nm and using the same equation described above. BSA protein has an extinction coefficient at 280 nm of 43 824 dm^3 mol^{-1} cm^{-1}.

Further fluorescent labelling experiments were performed with sulfoindocyanine dye Cy 5.0 (${}^{F}\varepsilon_{max} = 2.5 \times 10^5$ dm^3 mol^{-1} cm^{-1}) under the same conditions.

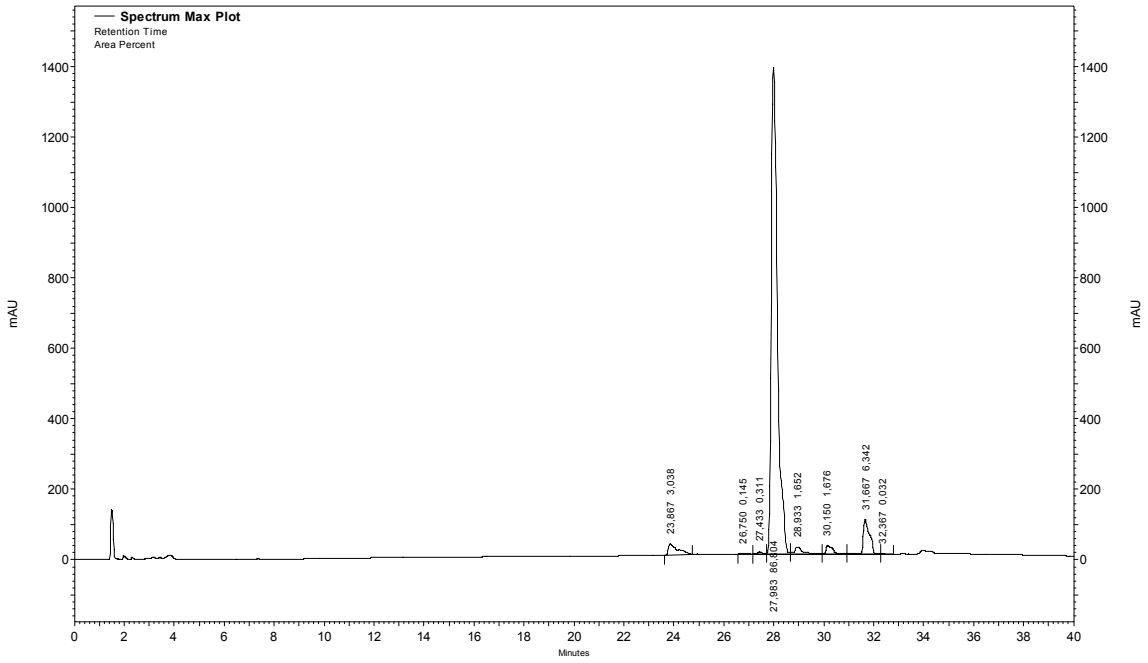
Normalised absorption (—), emission (—) and excitation (—) spectra of BODIPY 4 in CH_2Cl_2 at 25 °C.

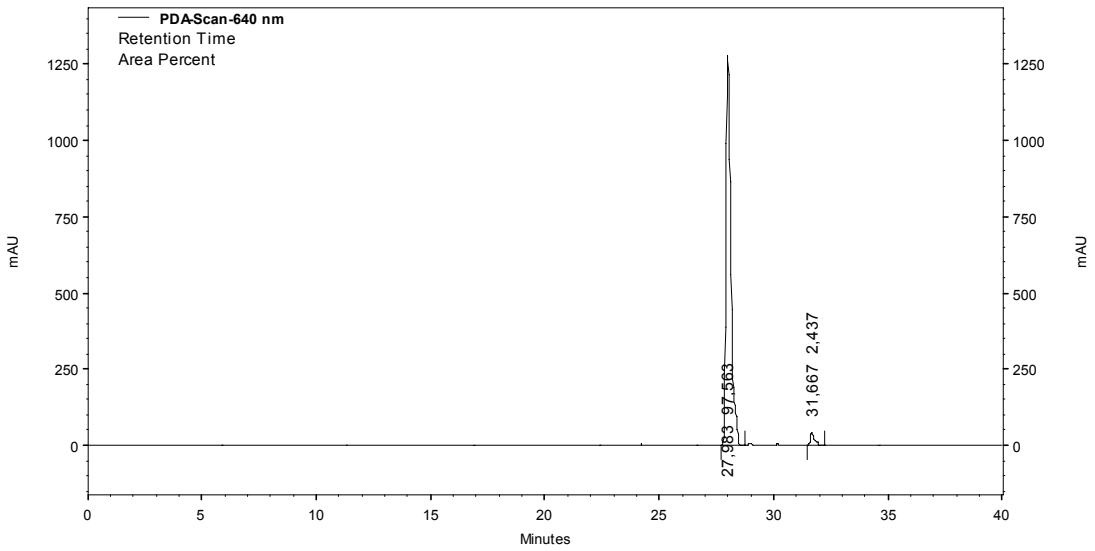


Normalised absorption (—), emission (—) and excitation (—) spectra of BODIPY 5 in CH_2Cl_2 at 25 °C.

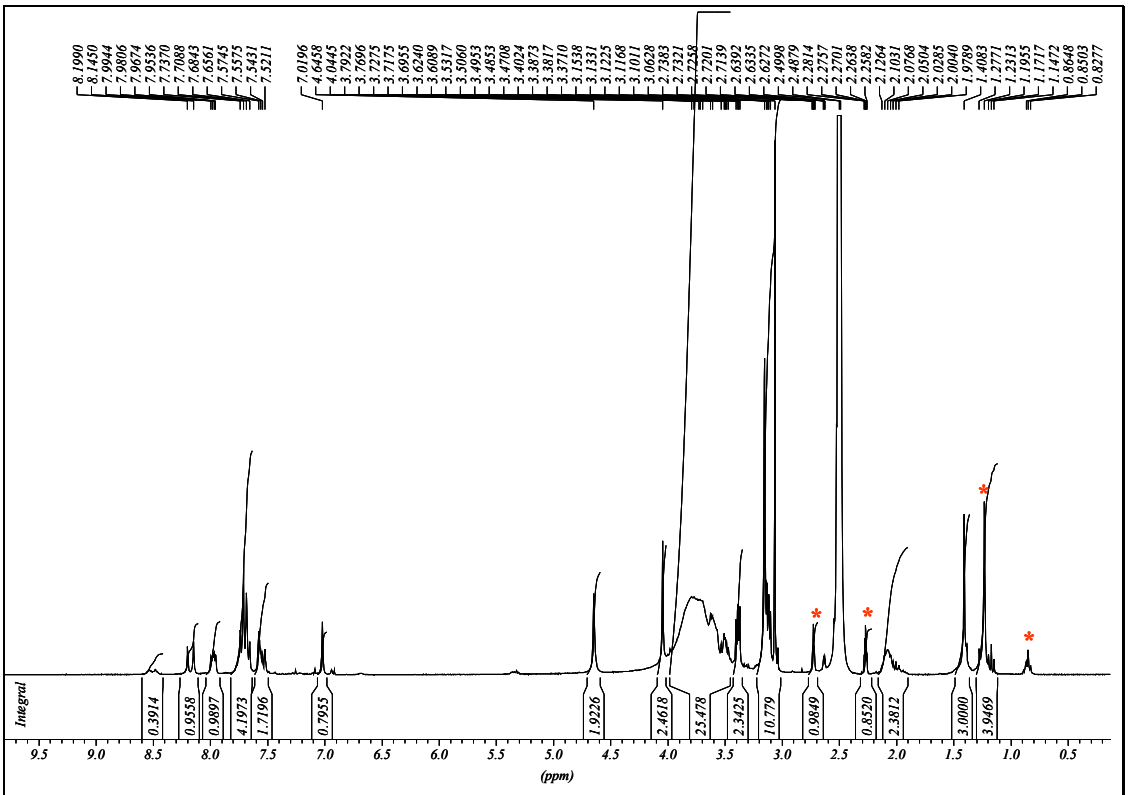


RP-HPLC elution profiles (system A, max plot 220-700 nm and 640 nm) of BODIPY carboxylic acid 8.



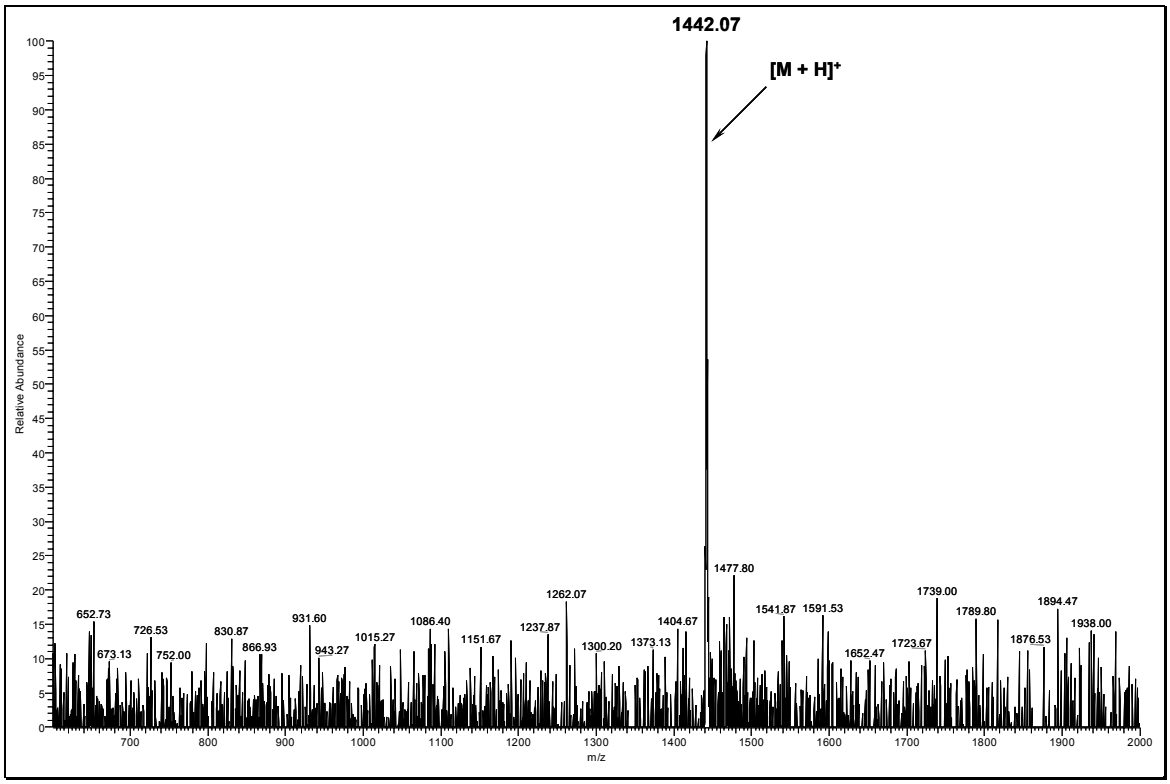


^{1}H NMR spectrum of BODIPY 9 recorded in DMSO-d_6.

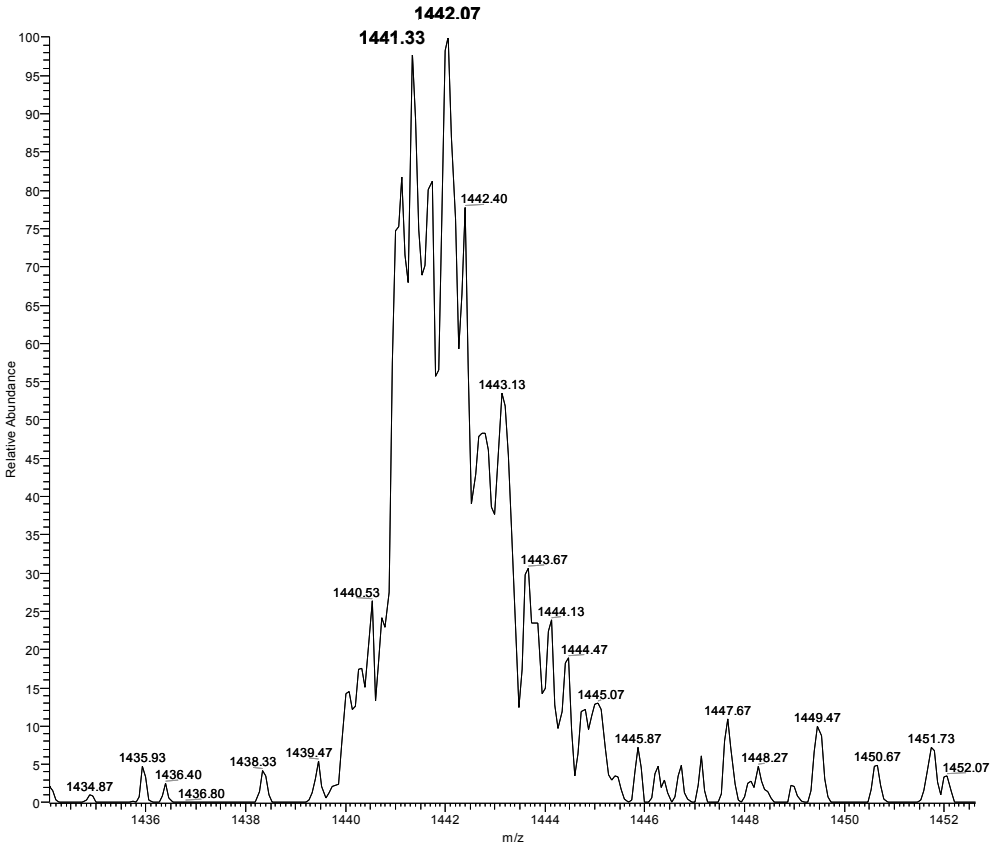


*peaks not assigned to protons of BODIPY, minor impurities recovered during desalting over Dowex resin.

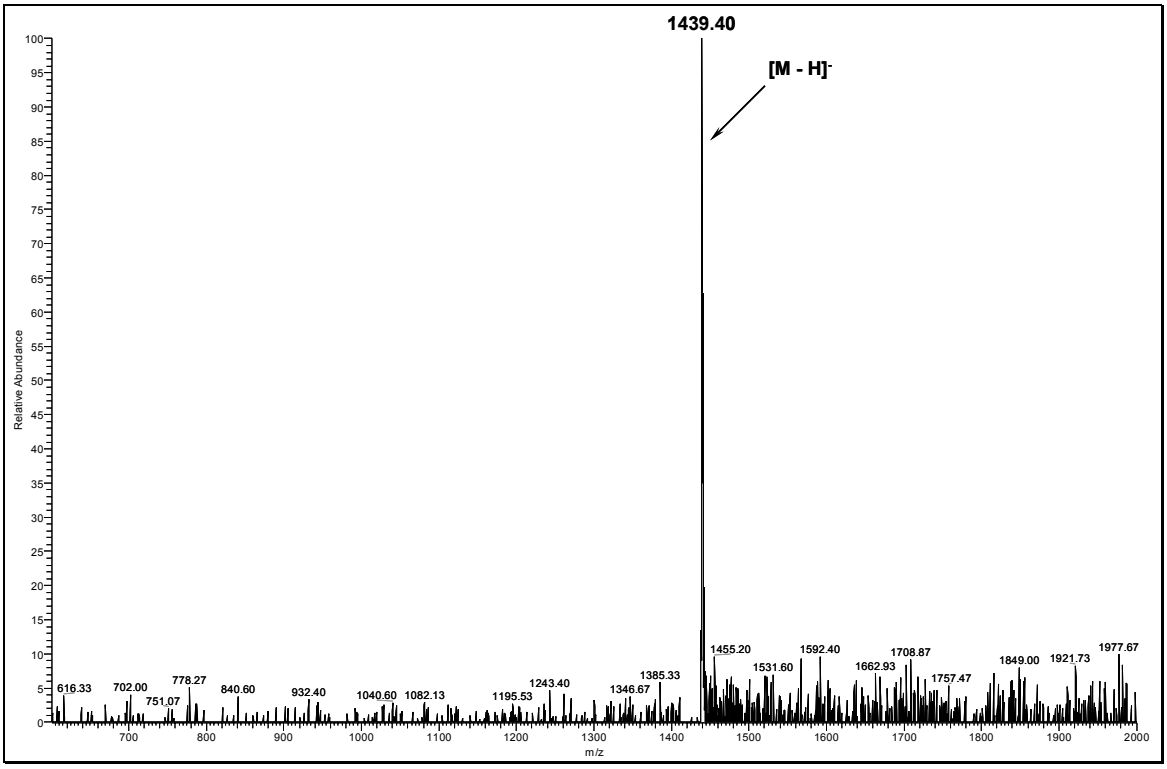
ESI-MS spectrum of BODIPY 9 recorded in the positive mode.



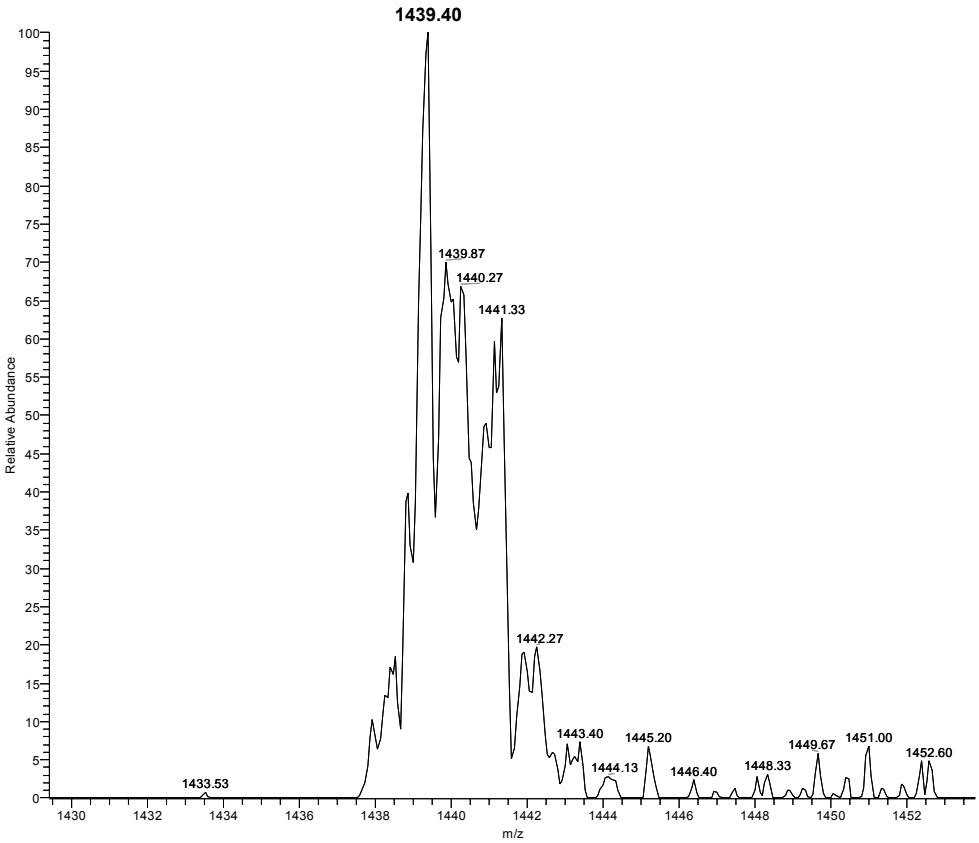
zoom-scan spectrum of $[M + H]^+$:



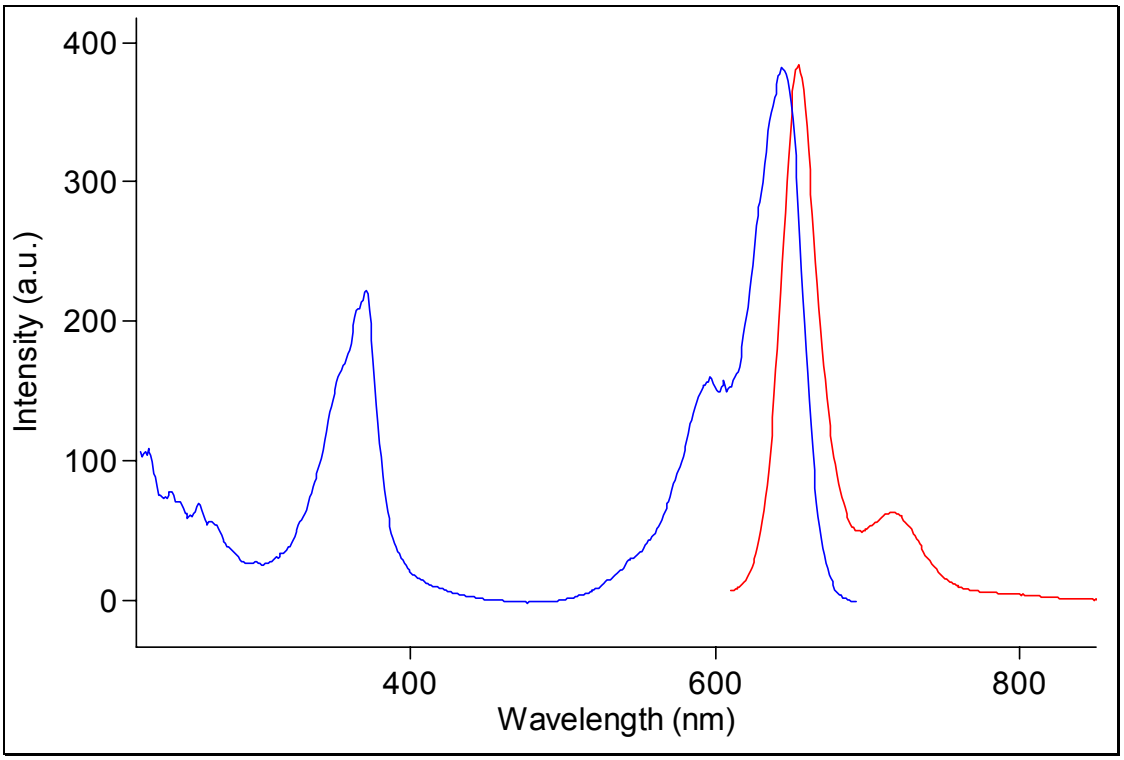
ESI-MS spectrum of BODIPY 9 recorded in the negative mode.



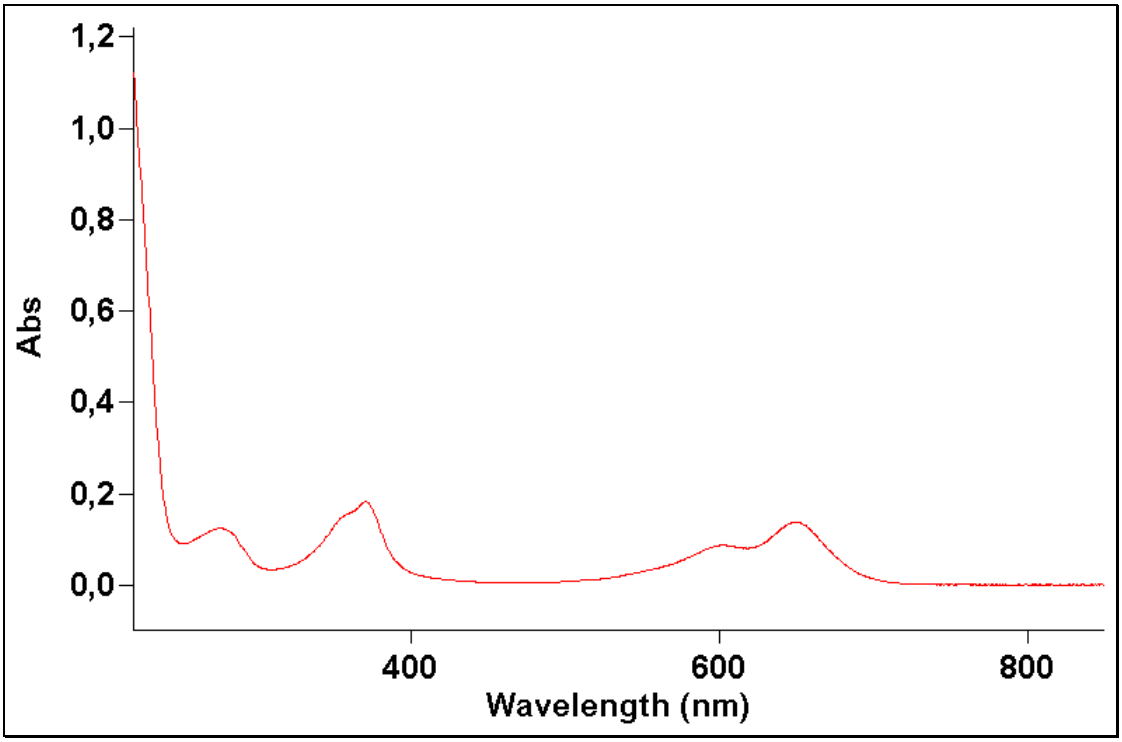
zoom-scan spectrum of $[M - H]^-$:



Normalised emission (—) (Ex. 600 nm) and excitation (—) (Em. 700 nm) spectra of water-soluble BODIPY 9 in PBS at 25 °C.

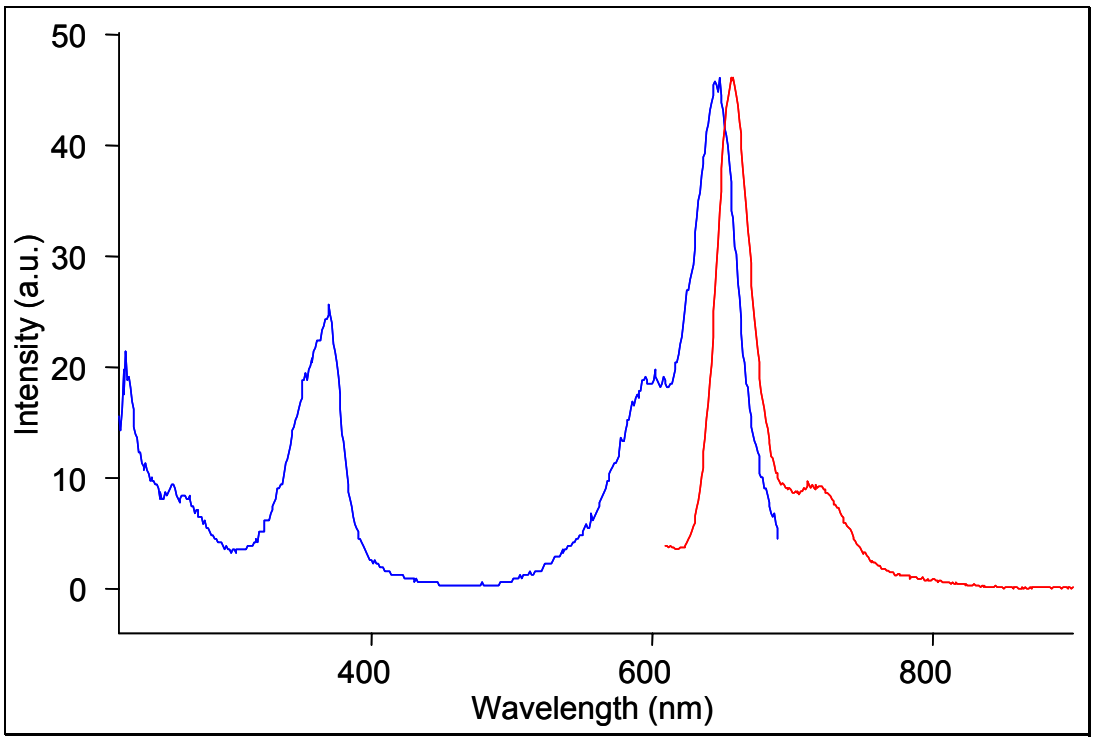


Absorption spectrum of BODIPY-mAb conjugate in PBS at 25 °C.

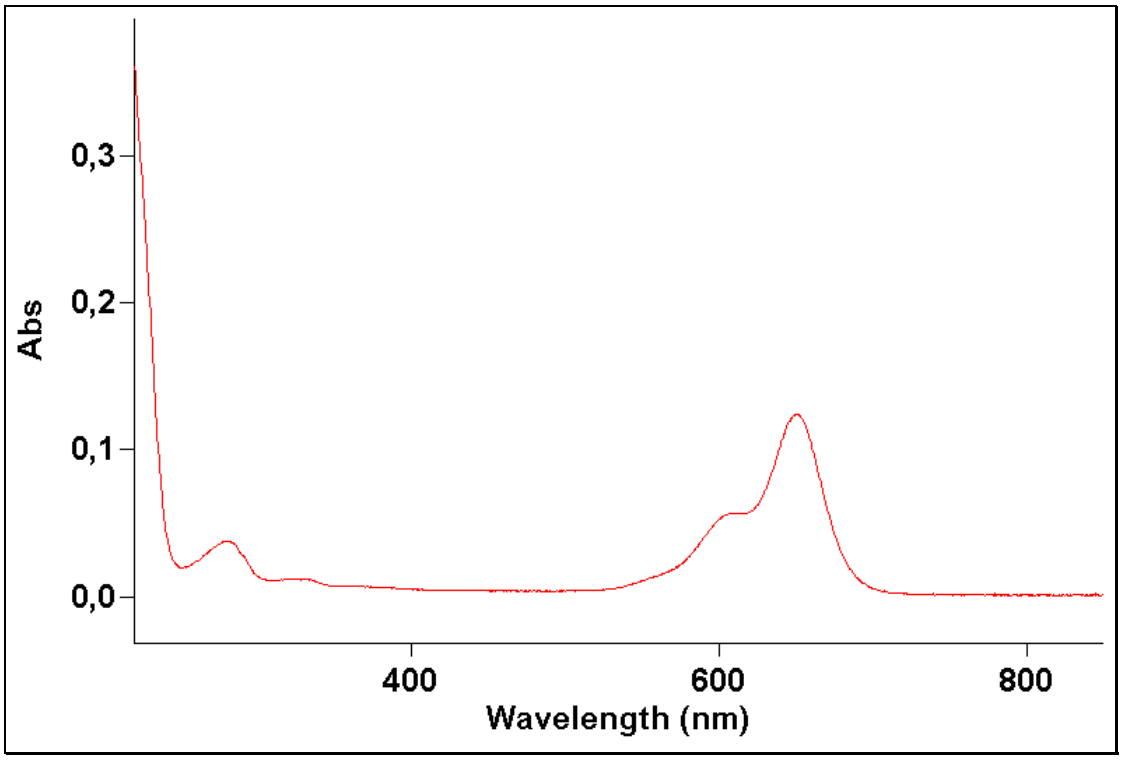


λ_{max}(PBS)/nm 276, 370, 603 and 649

Normalised emission (—) (Ex. 600 nm) and excitation (—) (Em. 700 nm) spectra of BODIPY-mAb conjugate in PBS at 25 °C.

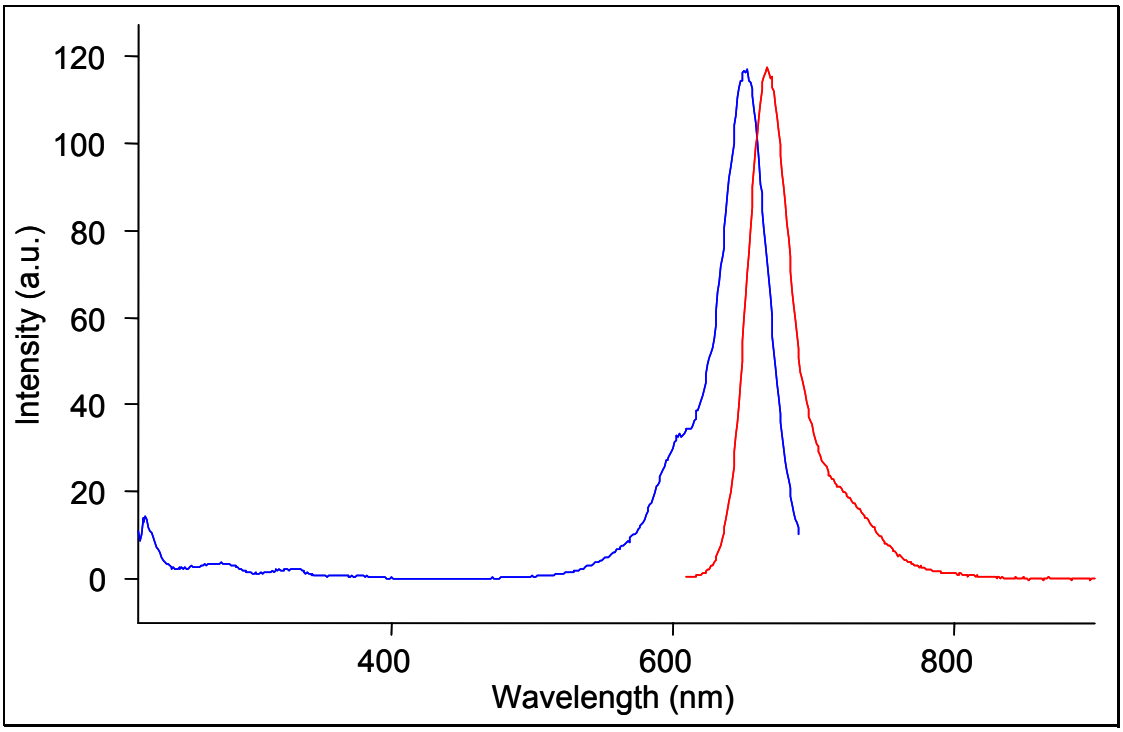


Absorption spectrum of Cy 5.0-mAb conjugate in PBS at 25 °C.

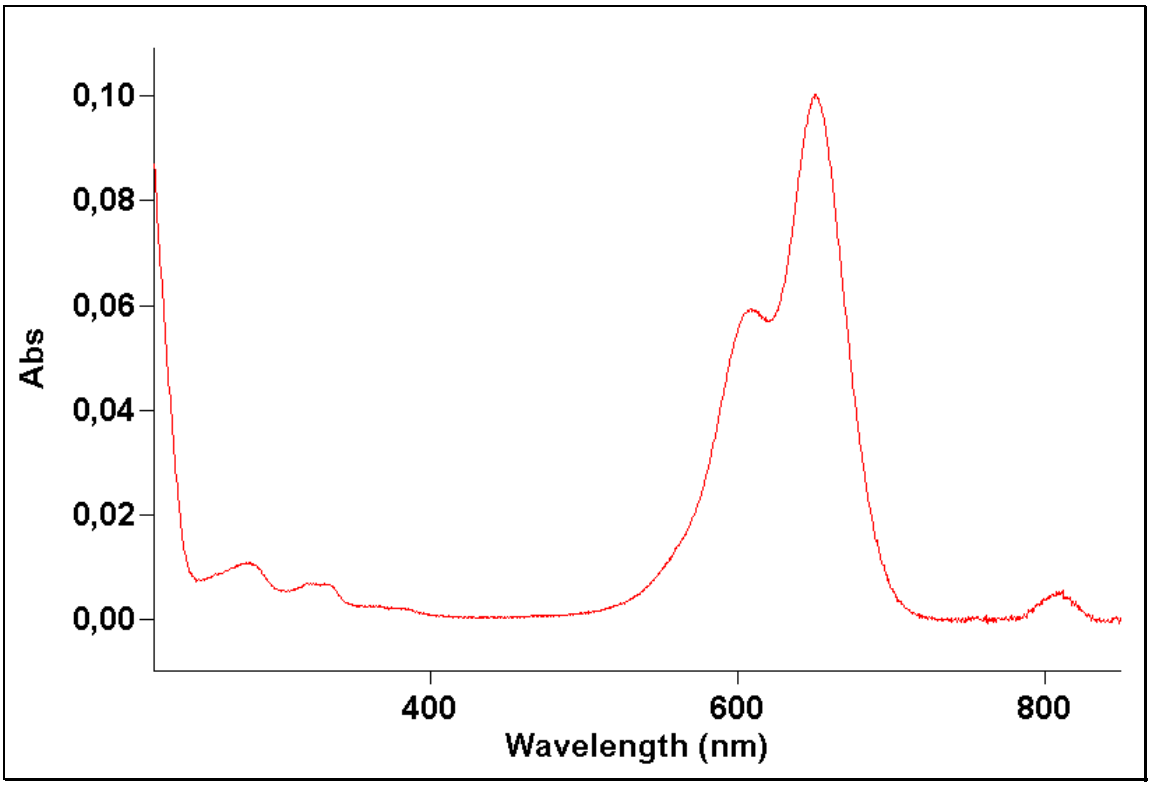


λ_{max}(PBS)/nm 281 and 650

Normalised emission (—) (Ex. 600 nm) and excitation (—) (Em. 700 nm) spectra of Cy 5.0-mAb conjugate in PBS at 25 °C.



Absorption spectrum of Cy 5.0-BSA conjugate in PBS at 25 °C.



λ_{max}(PBS)/nm 280, 609 and 650

Normalised emission (—) (Ex. 600 nm) and excitation (—) (Em. 700 nm) spectra of Cy 5.0-BSA conjugate in PBS at 25 °C.