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Electronic Supplementary Information

Enhanced Aqueous Solubility of Long Wavelength Voltage-Sensitive Dyes by Covalent Attachment of Polyethylene Glycol

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Full experimental details of synthesis and characterization of long wavelength voltage-sensitive dyes and intermediates are presented here.

Experimental

Chemical Syntheses: All reactions were carried out under an argon atmosphere in oven dried glassware. Unless specified, all reagents were purchased from either Aldrich Chemicals or ACROS Chemicals. Chromatographic material sources: silica gel TLC cards (Fluka 60778), RP-TLC plates (Analtech 52521), bulk silica gel (Aldrich 28,859-4), bulk reversed phase C_{18} silica gel (Separation Method Technologies BOD-35-150) and bulk C_2 silica gel (SMT BMEB2-35-150). mPEG-amine derivatizing reagents were obtained from Fluka (Sigma-Aldrich): O-(2-Aminoethyl)-O'-methylpolyethylene glycol-750 ($mPEG_{750}$-NH_2, Fluka 07964); O-(2-Aminoethyl)-O'-methylpolyethylene glycol-2,000 ($mPEG_{2000}$-NH_2, Fluka 06676); Polyoxyethylene bisamine (3,350 MW) (di-NH_2-PEG_{3400}, Sigma P9906-5G); and O-(2-Aminoethyl)-O'-methylpolyethylene glycol – 5,000 ($mPEG_{5000}$-NH_2, Fluka 06679).

Synthetic procedures for some of the intermediates used in the syntheses of the dyes reported here, namely 4-(2,3,3-trimethyl-5-sulfonato-indolinium-1-yl)butane-1-sulfonate **(6)**, 1-ethyl-(2,3,3-trimethyl-5-sulfonato-indolinium) bromide **(8)**, and 6-(2,3,3-trimethyl-3H-indolium-1-yl)hexanoic acid **(12)**, were previously reported by Mujumdar et al.[1] Further synthetic detail and analysis are presented here for intermediate **(4),** 4-(2,3,3-trimethyl-3H-indolium-1-yl)butane-1-sulfonate, previously described by Ernst et al.[2]

Dyes were characterised by NMR (300 or 500 MHz). Some dyes required the use of mixed solvent systems due to limited solubility in single solvents. Mass Spectra were obtained using a PerSeptive Voyager STR MS matrix assisted laser desorption ionization (MALDI) time of flight (TOF) mass spectrometer. Samples were prepared using equal volumes of analyte and matrix (10mg/mL alpha-cyano-4-hydroxycinnamic acid in 50:50 H2O:MeOH + 0.1% AcOH v/v). Calibration was done using a mixture of peptides (FMRF, Bradykinin, Angiotensin, Fibrinopeptide, Renin Substrate, ACTH) each at approx. 10uM in 50:50 H_2O:MeOH + 0.1% AcOH(v/v).

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SYNTHESIS OF INTERMEDIATES

N,N-dibutyl-4-ethenylaniline (1)
Butyllithium (2.5 M in hexane, 13.6 ml; 34.0 mmol) was added slowly to a solution of methyltriphenylphosphonium bromide (14.0 g; 39.2 mmol) in anhydrous THF (100 ml). Dibutylaminobenzaldehyde (5.0g; 21.6 mmol) was added and the reaction stirred under argon at room temperature. After 24 hours the reaction was poured into hexane (1000 ml) and stirred for 15 minutes. The yellow precipitate was removed by filtration through Celite. The filtrate was concentrated to approximately 20 ml by rotary evaporation and then passed through a 0.2 µm ultrafilter. Solvent removal by rotary evaporation produced a light yellow oil, which solidified on standing; yield 4.52g. (19.5 mmol, 90%). Silica gel TLC R_f = 0.8 (ethyl acetate-hexane,1:9). ^{1}H NMR ($CDCl_3$, 300 mHz): 0.897 (6 H, t, J = 7.1, -$CH_2CH_2CH_2CH_3$); 1.299 (4 H, m, -$CH_2CH_2CH_2CH_3$); 1.516 (4 H, m, -$CH_2CH_2CH_2CH_3$); 3.216 (4 H, t, J = 7.6, -$CH_2CH_2CH_2CH_3$); 4.915 (1 H, d, J = 11.1, -CH=CH*H*); 5.437 (1 H, d, J = 18.0, -CH=C*H*H); 6.548 (3 H, m, overlapping, -C*H*=CH_2, Phe); 7.221 (2 H, d, J= 6.4, Phe).

5-{(*E*)-2-[4-(dibutylamino)phenyl]ethenyl}thiophene-2-carboxaldehyde (2)
Freshly distilled 5-bromo-2-thiophene carboxaldehyde (1.91 mL,160.7 mmol) was mixed with dibutylaminovinylbenzene **(1)** (3.11g, 13.4 mmol), palladium (II) acetate (30.9 mg), tris-(o-tolyl)phosphine (84.7 mg) and triethylamine (6.15 ml) under argon in a thick-walled glass reaction vessel. The mixture was stirred magnetically and heated at 115-120 ^oC for 24 hours. The resulting deep brown mixture was cooled, stirred at room temperature overnight, then partitioned between methylene chloride (50 ml) and water (50 ml). The aqueous layer was washed with methylene chloride (2 x 40 ml). The organic layers were combined, dried over $MgSO_4$, and concentrated by rotary evaporation. Product residue was dissolved in 2:8 EtOAc: hexane (20 ml) and chromatographed over silica (80 g) eluted with a gradient of ethyl acetate in

hexane. The product was collected as a single component by silica gel TLC with R_f = 0.23 (ethyl acetate-hexane, 1:9); isolated yield of 2.75g (8.05 mmol, 60%). ^{1}H NMR ($CDCl_3$, 300 mHz): 0.994 (6 H, t, J = 7.3, -$CH_2CH_2CH_2CH_3$), 1.394 (4 H, m, -$CH_2CH_2CH_2CH_3$), 1.589 (4H, m, -$CH_2CH_2CH_2CH_3$), 3.257 (4H, t, J = 7.6, -$CH_2CH_2CH_2CH_3$), 6.580 (2 H, d, J = 8.9, -Phe), 6.996 (1 H, d, J = 16.3, =C*H*-); 7.061 (1 H, d, J = 3.9, -thiophene); 7.109 (1 H, d, J = 16.1, =C*H*-); 7.382 (2 H, d, J = 8.9, -Phe); 7.563 (1 H, d, J = 3.9, -thiophene); 9.755 (1 H, s, -C*H*O).

4-(4-methylquinolinium-1-yl)butane-1-sulfonate (3)

4-Methyl-quinoline (3 ml, 22.7 mmol) and 1,4-butanesultone (6.3 ml, 61.6 mmol) were heated at 100°C for 16 hours. The resulting solid was triturated with methanol-ethyl acetate (1:3) and recrystallised from methanol/ether yielding a light purple solid; yield 5.23g (18.7 mmol, 82%). RP-TLC R_f = 0.10 (methanol-water 1:9); ^{1}H NMR (DMSO-d_6 300 MHz) 1.958 (2 H, m, -$CH_2CH_2CH_2CH_2SO_3$); 2.274 (2 H, m, -$CH_2CH_2CH_2CH_2SO_3$); 2.923 (2 H, t, J = 7.2, -$CH_2CH_2CH_2CH_2SO_3$); 3.082 (3 H, s, -Me); 5.094 (2 H, t, J = 7.6, -$CH_2CH_2CH_2CH_2SO_3$); 7.971 (1 H, d, J = 5.6, Pyr); 8.067 (1 H, t, J = 7.6, -Phe); 8.283 (1 H, t, J = 8.1, -Phe); 8.580 (1 H, overlapping d, J = 8.2 -Phe); 8.619 (1 H, overlapping d, J = 8.8, -Phe); 9.268 (1 H, d, J = 6.5, Pyr).

4-(2,3,3-trimethyl-3H-indolium-1-yl)butane-1-sulfonate (4)

Briefly, 2,3,3-trimethyl-3H-indole (4.8 g) and 1,4-butanesultone (4.2 ml) were heated for 18 hours at 110°C. The resulting solid was triturated with ethyl acetate (50 ml). The product was collected by vacuum filtration and washed with ether (2 x 50 ml) yielding 9.5 g pink solid**.** RP-TLC R_f = 0.73 (acetonitrile-water, 6:4); ^{1}H NMR (D_2O, 300 MHz) – 1.519 (6 H, s, Ind-Me_2); 1.849 (2 H, m, -$CH_2CH_2CH_2CH_2SO_3$); 2.079 (2 H, m, -$CH_2CH_2CH_2CH_2SO_3$); 2.924 (2 H, t, J = 7.6, -$CH_2CH_2CH_2CH_2SO_3$); 4.476 (2 H, t, J = 7.8, -$CH_2CH_2CH_2CH_2SO_3$); 7.586 (2 H, overlapping, m, indolenine); 7.687 (1 H, m, indolenine); 7.755 (1 H, m, indolenine).

4-(2-methyl-1,3-benzothiazol-3-ium-3-yl)butane-1-sulfonate (5)

Similarly, 2-methylbenzothiazole (25 ml, 197 mmol), 1,4-butanesultone (18.1 ml, 177 mmol) and 1,2-dichlorobenzene (100 ml, anhydrous) were heated overnight at 110°C. After cooling the mixture was diluted with ether (350 ml). The product was collected by vacuum filtration, washed with ether (2 x 100 ml), then dried *in vacuo*, yielding a crystalline solid, 6.87 g (24.1 mmol, 14%) showing a single component by RP-TLC (water) R_f = 0.28. E 210 nm = 10,921 $M^{-1}cm^{-1}$; ^{1}H NMR (D_2O, 300 MHz) – 1.889 (2 H, m, -$CH_2CH_2CH_2CH_2SO_3$); 2.083 (2 H, m, -$CH_2CH_2CH_2CH_2SO_3$); 2.938 (2 H, t, J = 7.6, -$CH_2CH_2CH_2CH_2SO_3$); 3.141 (3 H, s, -Me); 4.715 (obscured by D_2O, -$CH_2CH_2CH_2CH_2SO_3$); 7.728 (1 H, t, J = 7.4, Phe); 7.833 (1 H, t, J = 7.4, Phe); 8.115 (2 H, overlapping doublets, J = 11.0, J = 10.5, Phe).

4-(6-methoxy-2-methylquinolinium-1-yl)butane-1-sulfonate (9)

1,4-Butanesultone (1.08ml, 10.5mmol) was added to a solution of 6-methoxyquinaldine (2.0g, 11.55 mmol) in tetramethylene sulfone (5.0 ml). After heating at 110 °C for 3 days, the mixture was cooled and diluted with ethyl acetate (20 ml). The resulting solid was collected on a vacuum funnel and washed with diethyl ether (2x10 ml), ethyl acetate (10 ml) and dried under vacuum. C_{18}-RP-TLC (MeOH-H_2O, 1:1) gave R_f = 0.50. Yield: 1.86g (6.01 mmol, 52%). ^{1}H NMR (DMSO-d_6, 300 mHz): 1.897 (2 H, m, -$CH_2CH_2CH_2CH_2SO_3$); 2.034 (2 H, m, -$CH_2CH_2CH_2CH_2SO_3$); 2.663 (2 H, t, J = 7.0, -$CH_2CH_2CH_2CH_2SO_3$); 3.055 (3 H, s, -Me); 3.98 (3 H, s, -OMe); 4.886 (2 H, t, J = 8.2, -$CH_2CH_2CH_2CH_2SO_3$); 7.736-7.838 (2 H, m, overlapping, Phe, Phe); 7.983 (1 H, d, J = 8.3, Phe); 8.527 (1 H, d, J = 9.5, Phe); 8.883 (1 H, d, J = 9.0, Pyr).

4-(6-methoxy-4-methylquinolinium-1-yl)butane-1-sulfonate (10)

6-Methoxy-4-methylquinoline, (1.0g, 5.77 mmol) dissolved in tetramethylene sulfone (2.5 ml) was mixed with 1,4-butanesultone (0.54ml, 5.27mmol) and heated at 110 °C for 3 days. After cooling, the reaction was diluted with ethyl acetate (20 ml). The resulting solid was collected, washed with ether (2 x 10 ml) and ethyl acetate (10 ml), and dried *in vacuo*. RP-TLC (MeOH-H_2O, 1:1) R_f = 0.50 Yield 1.50g (4.85 mmol, 84.0%). ^{1}H NMR (D_2O, 300 mHz): 1.805 (2 H, m, -$CH_2CH_2CH_2CH_2SO_3$); 2.154 (2 H, m, -$CH_2CH_2CH_2CH_2SO_3$); 2.876-2.183 (5 H, overlapping (s+t), J = 7.4, -Me, -$CH_2CH_2CH_2CH_2SO_3$); 3.987 (3 H, s, -OMe); 4.910 (2 H, t, J = 7.3, -$CH_2CH_2CH_2CH_2SO_3$); 7.542 (1 H, d, J = 3.1, Phe between -Me and -OMe); 7.732 (1 H, overlapping, s, Phe); 7.774 (1 H, overlapping, d, J = 6.1, Pyr); 8.268 (1 H, d, J = 10.1, Phe); 8.883 (1 H, d, J = 6.0, Pyr).

1-(5-carboxypentyl)-4-methylquinolinium bromide (11A) (Isolated as COOH): Lepidine (4.80 ml, 0.0363 mol) and 6-bromohexanoic acid (35.4g, 0.182 mol) were heated 110°C overnight. After cooling, the mixture was diluted with water (200 ml) and extracted with ethyl acetate (200 ml). The aqueous layer was washed with dichloromethane (2 x 50 ml) and then concentrated by rotary evaporation. The resulting oily residue was dissolved in 15 ml of methanol and crystallised upon addition of ethyl acetate, yielding 6.1g (0.0180 mol, 50.0%). C_{18}-RP-TLC (MeOH-H_2O, 1:1) R_f = 0.30 ^{1}H NMR (D_2O, 300 mHz): 1.383 (2 H, m, -$CH_2CH_2CH_2CH_2COOH$), 1.589 (2 H, m, -$CH_2CH_2CH_2CH_2COOH$), 2.004 (2 H, m, -$CH_2CH_2CH_2CH_2COOH$), 2.301 (2 H, t, J = 7.1, -$CH_2CH_2CH_2CH_2COOH$), 2.955 (3 H, s, -Me), 4.921 (2 H, t, J = 7.6, -$CH_2CH_2CH_2CH_2COOH$), 7.813 (1 H, d, J = 6.3, Pyr), 7.946 (1 H, t, J = 7.4, Phe), 8.147 (1 H, t, J = 7.3, Phe), 8.332 (1 H, d, J =8.7, Phe), 8.422 (1 H, d, J = 8.3, Phe), 8.982 (1 H, d, J = 5.7, Pyr).

(11B) (Isolated as DIPEA salt): Lepidine (15 ml, 113 mmol), 6-bromohexanoic acid (22.07 g, 113 mmol), and diisopropylethyl amine (14.6 g, 113 mmol) were mixed and heated at 120 °C for 18 hours. Reaction progress was monitored by C_{18}-RPTLC (MeOH-H_2O, 1:1, R_f 0.35). After cooling, the mixture was diluted with water (200 ml) and extracted with dichloromethane (200 ml). The aqueous layer

was made alkaline (pH 8-9) using 20 ml of saturated sodium bicarbonate solution followed by extraction with 250 ml of dichloromethane. The organic layer was separated and washed with water (100 ml). Aqueous layers were combined and dried by rotary evaporation. Product was purified by flash chromatography (C_{18}-RP) eluting with a methanol in water gradient (0 to 20%). Fractions containing pure DIPEA salt were combined and dried yielding 23.37 g of a hygroscopic solid (44%). ^{1}H NMR (D_2O, 300 mHz): 1.302 (15 H, m, DIPEA $-CH_3$); 1.375 (2 H, m, $-CH_2CH_2CH_2CH_2CH_2COOH$), 1.595 (2 H, m, $-CH_2CH_2CH_2CH_2CH_2COOH$), 2.029 (2 H, m, $-CH_2CH_2CH_2CH_2CH_2COOH$), 2.200 (2 H, t, J = 7.3, $-CH_2CH_2CH_2CH_2CH_2COOH$), 3.178 (2 H, m, DIPEA $-H\{CH_3\}_2$); 3.694 (2 H, m, DIPEA $-CH_2CH_3$); 4.912 (2 H, t, J = 7.3, $-CH_2CH_2CH_2CH_2CH_2COOH$), 7.829 (1 H, d, J = 6.5, Pyr), 7.931 (1 H, t, J = 7.6, Phe), 8.147 (1 H, t, J = 7.9, Phe), 8.328 (1 H, d, J =9.0, Phe), 8.397 (1 H, d, J = 8.9, Phe), 8.995 (1 H, d, J = 6.3, Pyr).

SYNTHESIS OF DYES & DERIVATIVES

Dyes (PGH-1 through PGH-10) were synthesised by the following general method. Equi-molar quantities of anilino-thiophene-carboxaldehyde **(2)** in ethanol (~0.25M) and quaternized heterocyclic intermediate **(3** through **12)** dissolved in methanol (~0.25M) were stirred at room temperature. Quinoline and Lepidine intermediates **(3**, **9**, **10** and **11)** required the addition of 1% (v/v) piperidine to facilitate the reaction. At various times a sample of the mixture was diluted in ethanol and measured by UV/Vis spectroscopy. Reaction progress was monitored by appearance of the product dye (Table 3) and decrease of the aldehyde (A_{444}). If significant starting aldehyde remained unreacted, additional heterocycle was added and the reaction continued. When the A_{dye} / A_{444} ratio remained constant, the reaction was concentrated to dryness by rotary evaporation. Dyes were purified by either normal or reversed phase column chromatography.

4-{4-[(*E*)-2-(5-{(*E*)-2-[4-(dibutylamino)phenyl]ethenyl}-thiophen-2-yl)ethenyl]quinolinium-1-yl}butane-1-sulfonate (PGH-1)

Intermediates **(2)** and **(3)** (7.17 mmol) were condensed with the aid of piperidine to form the product dye which was purified by column chromatography over silica gel (75g), and eluted with a methanol-ethyl acetate gradient. Fractions containing pure PGH 1 dye [silica TLC (MeOH-EtOAc, 1:1); R_f = 0.20] were combined and dried by rotary evaporation. Yield 1.3g (2.16 mmol, 30%). λ_{max}(MeOH)/nm 602 (ε/dm^3 mol^{-1} cm^{-1} 24 895). ^{1}H NMR (DMSO-d_6, 300 mHz): 0.926 (6 H, t, J = 7.4, $-CH_2CH_2CH_2CH_3$); 1.271-1.395 (4 H, m, $-CH_2CH_2CH_2CH_3$); 1.469-1.544 (4 H, m, $-CH_2CH_2CH_2CH_3$); 1.684-1.734 (2 H, m, $-CH_2CH_2CH_2CH_2SO_3$); 2.035-2.082 (2 H, m $-CH_2CH_2CH_2CH_2SO_3$); 2.541 (obscured by DMSO-d_6 signal, $-CH_2CH_2CH_2CH_2SO_3$); 3.338 (obscured by H_2O signal, $-CH_2CH_2CH_2CH_3$); 4.950 (2 H, t, J = 7.01, $-CH_2CH_2CH_2CH_2SO_3$); 6.656 (2 H, d, J = 8.5, Phe); 7.016 (1 H, d, J = 16.2, -CH=); 7.180-7.231 (2 H, overlapping signals s+d, J = 4.3 and 15.3, thiophene, -CH=); 7.422 (2 H, d, J = 8.9, Phe); 7.644 (1 H, d, J = 4.2, thiophene); 7.830 (1 H, d, J = 15.6, -CH=); 7.992 (1 H, t, J = 7.5, Phe); 8.211 (1 H, t, J = 7.5, Phe); 8.358-8.405 (2 H, t, J = 7.5 and 6.5, Phe, Pyr); 8.565 (1 H, d, J = 8.5 Phe); 8.909 (1 H, d, J = 8.5 Phe); 9.282 (1 H, d, J = 6.5, Pyr). MALDI-TOF *m/z* 603 (M+, 100%), 625 (84).

4-{2-[(*E*)-2-(5-{(*E*)-2-[4-(dibutylamino)phenyl]ethenyl}-thiophen-2-yl)ethenyl]-3,3-dimethyl-3*H*-indolium-1-yl}butane-1-sulfonate (PGH-2)

The dye made from intermediates **(2)** and **(4)** (1.18 mmol) was separated over C_{18}-reversed phase silica gel eluting with a step gradient of methanol in water (10% steps, 100 ml/step). Fractions containing pure PGH-2 dye [C_{18}-RP TLC (MeOH-water, 6:4); R_f = 0.20] were combined and dried, yielding 0.22 g (0.36 mmol, 30%). λ_{max}(iPrOH)/nm 670 (ε/dm^3 mol^{-1} cm^{-1} 33 760). ^{1}H NMR (DMSO-d_6, 300 mHz): 0.931 (6 H, t, J = 7.4, $-CH_2CH_2CH_2CH_3$); 1.303-1.400 (4 H, m, $-CH_2CH_2CH_2CH_3$); 1.478-1.576 (4 H, m, $-CH_2CH_2CH_2CH_3$); 1.765 (6 H, s, Ind-Me_2); 1.802-1.849 (2 H, m, $-CH_2CH_2CH_2CH_2SO_3$); 1.849-1.972 (2 H, m, $-CH_2CH_2CH_2CH_2SO_3$); 2.559 (2 H, t, J = 7.1, $-CH_2CH_2CH_2CH_2SO_3$); 3.333 (obscured by D_2O signal, $-CH_2CH_2CH_2CH_3$); 4.561 (2 H, t, J = 7.2, $-CH_2CH_2CH_2CH_2SO_3$); 6.681 (2 H, d, J = 8.7, Phe); 7.140 (1 H, d, J = 15.7, -CH=); 7.279 (2 H, d, J = 2.0, -HC=CH-); 7.387 (1 H, d, J = 3.9, thiophene); 7.494 (2 H, d, J = 8.7, Phe); 7.534-7.584 (2 H, dd, J = 6.8 and 1.4, Ind); 7.805 (1 H, d, J = 7.3, Ind), 7.873 (1 H, d, J = 7.7, Ind); 8.140 (1 H, d, J = 4.0, thiophene); and 8.604 (1 H, d, J = 15.6, -CH=).

4-{2-[(*E*)-2-(5-{(*E*)-2-[4-(dibutylamino)phenyl]ethenyl}-thiophen-2-yl)ethenyl]-1,3-benzothiazol-3-ium-3-yl}-butane-1-sulfonate (PGH-3)

Intermediates **(2)** and **(5)** (2.34 mmol) were combined to form a blue product. The residue was dissolved in a mixture of methanol (8 ml), trifluoroacetic acid (0.36 ml), and dichloromethane (70 ml), and separated over a silica gel column (30 g) eluting with a methanol in dichloromethane gradient. Fractions containing pure dye (silica TLC developed with methanol-ethyl acetate, 1:1, R_f = 0.56) were combined and dried. The resulting film was dissolved in methanol-dichloromethane (1:3) and precipitated with hexane (100 ml). Product was collected by filtraton and dried *in vacuo* yielding 0.54 g of blue powder (0.89 mmol, 38%). λ_{max}(MeOH)/nm 614 (ε/dm^3 mol^{-1} cm^{-1} 47 384). ^{1}H NMR (CDCl3 with 10% MeOH-d_4, 300 mHz): 0.917 (6 H, *t*, J = 7.3, $-CH_2CH_2CH_2CH_3$); 1.261-1.384 (4 H, m, $-CH_2CH_2CH_2CH_3$); 1.481 -1.582 (4 H, m, $-CH_2CH_2CH_2CH_3$); 2.029-2.143 (4 H, overlapping m, $-CH_2CH_2CH_2CH_2SO_3$); 2.997 (2 H, t, J = 6.3, $-CH_2CH_2CH_2CH_2SO_3$); 3.270 (4 H, t, J = 7.7, $-CH_2CH_2CH_2CH_3$); 4.680 (2 H, t, J = 7.4, $-CH_2CH_2CH_2CH_2SO_3$); 6.616 (2 H, d, J = 8.6, Phe); 6.816 (1 H, d, J = 15.9, -CH=); 6.918-6.992 (2 H, overlapping m, -CH=, Phe); 7.259 – 7.312 (3 H, overlapping m, -CH=CH-, thiophene); 7.469 (1 H, t, J = 7.62, -CH=), 7.608 (1 H, t, J = 7.6, Benzth); 7.650 (1 H, d, J = 3.8, thiophene); 7.841-7.905 (3 H, m, overlapping, Benzth).

Potassium-2-[(*E*)-2-(5-{(*E*)-2-[4-(dibutylamino)phenyl]-ethenyl}thiophen-2-yl)ethenyl]-3,3-dimethyl-1-(4-sulfonatobutyl)-3*H*-indolium-5-sulfonate (PGH-4)

The dye resulting from reacting **(2)** with intermediate **(6)** (8.11 mmol) was purified by chromatography over C_{18}-reversed phase silica gel (175g) equilibrated with water and eluted with a methanol-water step gradient from 0 to 65%. Fractions containing pure dye (C_{18}-RP-TLC, AcN-H2O 4:6, R_f = 0.23) were combined and dried giving 2.2 g blue solid. This residue was dissolved in 70 ml dimethylsulfoxide and precipitated with ethyl acetate (700 mL). Pure PGH-4 dye was recovered by filtration and vacuum dried. Yield: 1.5 g (2.03 mmol, 25.2%). λ_{max}(EtOH)/nm 690 (ε/dm^3 mol^{-1} cm^{-1} 55 172). ^{1}H NMR (DMSO-d_6, 300 mHz): 0.930 (6 H, t, J = 7.3, -$CH_2CH_2CH_2CH_3$), 1.338 (4 H, m, -$CH_2CH_2CH_2CH_3$), 1.525 (4 H, m, -$CH_2CH_2CH_2CH_3$), 1.770 (6 H, s, Ind-*Me*$_2$), 1.814-1.910 (4 H, overlapping m, -$CH_2CH_2CH_2CH_2SO_3$), 2.585 (2 H, m, -$CH_2CH_2CH_2CH_2SO_3$), 3.35 (4 H, obscured by D_2O, -$CH_2CH_2CH_2CH_3$), 4.539 (2 H, t, J = 6.8, -$CH_2CH_2CH_2CH_2SO_3$), 6.679 (2 H, d, J = 9.1, Phe), 7.122 (1 H, d, J = 15.6, -CH=), 7.285 (2 H, overlapping m, -*H*C=C*H*-), 7.387 (1 H, d, J = 4.2 , thiophene), 7.498 (2 H, d, J = 8.6, Phe), 7.799 (2 H, s, Ind), 8.002 (1 H, s, Ind), 8.145 (1 H, d, J = 4.5, thiophene), 8.624 (1 H, d, J = 15.5, =C*H*-).

Potassium iodide - 2-[(*E*)-2-(5-{(*E*)-2-[4-(dibutylamino)-phenyl]ethenyl}thiophen-2-yl)ethenyl]-1-ethyl-3,3-dimethyl-3*H*-indolium-5-sulfonate (1:1) (PGH-6)

Intermediates **(2)** and **(8)** (1.18 mmol) formed a dye which was purified over C_{18}-reversed phase silica (50g) equilibrated with water and eluted with a 2-propanol-water gradient (0 to 75%). Fractions containing pure dye (C_{18}-RPTLC (IPA-H_2O, 1:1) R_f = 0.30) were combined and dried giving 0.49 g (0.66 mmol 56% yield) blue solid. λ_{max}(EtOH)/nm 690 (ε/dm^3 mol^{-1} cm^{-1} 67 577). ^{1}H NMR (DMSO-d_6, 300 mHz): 0.934 (6 H, t, J = 7.4, -$CH_2CH_2CH_2CH_3$); 1.306-1.447 (7 H, m, overlapping -$CH_2CH_2CH_2CH_3$, -CH_2CH_3); 1.480-1.558 (4 H, m, -$CH_2CH_2CH_2CH_3$); 1.785 (6 H, s, Ind-*Me*$_2$); 3.365 (obscured by DMSO-d_6 signal, -$CH_2CH_2CH_2CH_3$); 4.562 (2 H, m, -CH_2CH_3); 6.689 (2 H, d, J = 9.3, Phe), 7.030 (1 H, d, J = 15.3, -CH=); 7.207-1.339 (2 H, dd, J = 15.6 and 8.4, -C*H*=C*H*-); 7.389 (1 H, d, J = 4.2, thiophene); 7.490 (2 H, d, J = 9.0, Phe); 7.748-7.832 (2 H, dd, J = 8.2 and 8.0, Ind); 8.024 (1 H, s, Ind); 8.105 (1 H, d, J = 4.1, thiophene); 8.649 (1 H, d, J = 15.5, =CH-).

4-{2-[(*E*)-2-(5-{(*E*)-2-[4-(dibutylamino)phenyl]ethenyl}-thiophen-2-yl)ethenyl]-6-methoxyquinolinium-1-yl}butane-1-sulfonate (PGH-7)

The reaction products from intermediates **(2)** and **(9)** (0.589 mmol) with piperidine were separated by silica gel chromatography eluting with an acetonitrile-dichloromethane chloride gradient (0 to 100%). Pure PGH-7 dye (0.13 g, 0.205 mmol, 34.8%) was recovered showing R_f = 0.59 by silica gel TLC (MeOH/EtOAc, 1:1). λ_{max}(EtOH)/nm 594 (ε/dm^3 mol^{-1} cm^{-1} 42 124). ^{1}H NMR (1:3 MeOH-d_4:$CDCl_3$, 300 mHz): 1.046 (6 H, t, J = 7.2, -$CH_2CH_2CH_2CH_3$); 1.388-1.1.510 (4 H, m, -$CH_2CH_2CH_2CH_3$); 1.569-1.725 (4 H, m, -$CH_2CH_2CH_2CH_3$); 2.180-2.364 (4 H, broad m, -$CH_2CH_2CH_2CH_2SO_3$); 3.150 (2 H, t, J = 6.0, -$CH_2CH_2CH_2CH_2SO_3$); 3.378 (obscured by MeOH signal, t, J = 7.7, -$CH_2CH_2CH_2CH_3$); 3.928 (3 H, s, -OMe); 4.860 (2 H, broad, -$CH_2CH_2CH_2CH_2SO_3$); 6.656 (2 H, d, J = 9.0, Phe); 6.806 (1 H, d, J = 16.4, -CH=); 6.916-6.972 (2 H, overlapping signals s+d, J = 16.6, thiophene, -CH=); 7.037 (1 H, d, J = 14.7, -CH=); 7.285 (1 H, s, Phe); 7.353 (2 H, d, J = 8.4, Phe); 7.548 (1 H, d, J = 3.2, thiophene); 7.661 (1 H, t, J = 10.2 Phe); 8.061 (1 H, d, J = 15.3 -CH=); 8.250 (2 H, d, J = 9.3 Phe); 8.488 (1 H, d, J = 8.5, Phe).

4-{4-[(*E*)-2-(5-{(*E*)-2-[4-(dibutylamino)phenyl]ethenyl}-thiophen-2-yl)ethenyl]-6-methoxyquinolinium-1-yl}butane-1-sulfonate (PGH-8)

The dye from intermediates **(2)** and **(10)** (0.589 mmol) and piperidine was purified by silica gel chromatography eluting with a methanol-dichloromethane gradient yielding 95 mg PGH-8 dye (0.150 mmol, 25%) showing a single component by C_{18}-RP-TLC (IPA-H_2O, 3:1; R_f = 0.67). λ_{max}(EtOH)/nm 598 (ε/dm^3 mol^{-1} cm^{-1} 34 056). ^{1}H NMR (10% MeOH-d_4/90% $CDCl_3$, 500 mHz): 0.893 (6 H, t, J = 7.0, -$CH_2CH_2CH_2CH_3$); 1.264-1.322 (4 H, m, -$CH_2CH_2CH_2CH_3$); 1.520 (4 H, broad, -$CH_2CH_2CH_2CH_3$); 1.923 (2 H, m, -$CH_2CH_2CH_2CH_2SO_3$); 2.186 (2 H, m, -$CH_2CH_2CH_2CH_2SO_3$); 2.919 (2 H, t, J = 7.0, -$CH_2CH_2CH_2CH_2SO_3$); 3.247 (4 H, broad, -$CH_2CH_2CH_2CH_3$); 4.010 (3 H, s, -OMe); 4.885 (2 H, t, J = 7.3, -$CH_2CH_2CH_2CH_2SO_3$); 6.564 (2 H, broad, Phe(2)); 6.928 (3 H, broad signal,-CH=, -CH=, thiophene); 7.261-7.299 (4 H, m (broad), Phe, Phe, thiophene -CH=); 7.570 (1 H, s, Phe); 7.709 (1 H, d, J = 9.7, Phe); 7.898 (1 H, d, J = 15.3 –CH=); 7.970 (1 H, d, J = 5.24 Pyr); 8.220 (1 H, d, J = 9.8 Phe); 8.985 (1 H, broad, Pyr).

1-(5-carboxypentyl)-4-[(*E*)-2-(5-{(*E*)-2-[4-(dibutylamino)-phenyl]ethenyl}thiophen-2-yl)ethenyl]quinolinium bromide (PGH-9)

Method A- COOH form: Solutions of Carboxaldehyde **(2)** and quaternized lepidine **(11A)** (2.51 mmol) and were mixed with piperidine and stirred overnight at ambient temperature. When the A_{612} / A_{398} ratio remained constant, the mixture was concentrated to dryness, and purified by silica chromatography, eluting with a methanol–dichloromethane gradient [TLC MeOH-EtOAc (3:1), R_f = 0.25]. Yield: 0.335 g (0.506 mmol, 34%).

Method B- DIPEA salt and conversion to COOH: Carboxaldehyde **(2)** and quaternized lepidine **(11B)** (3 mmol) solutions were stirred with piperidine. Product was purified by chromatography as described above giving 2.08 g blue solid. The DIPEA salt was converted to the free carboxylic acid by partitioning between dichloromethane (130ml) and 10% hydrobromic acid (125 ml). The organic layer was separated, washed with water and dried giving 0.66 g PGH-9 pure by TLC (methanol-ethyl acetate, 3:1, R_f 0.25) (0.997 mmol, 33%).

^{1}H NMR (AcN-d_3, 300 mHz): 0.952 (6 H, t, J = 7.3, -$CH_2CH_2CH_2CH_3$); 1.318-1.393 (4 H, m, -$CH_2CH_2CH_2CH_3$); 1.417-1.465 (2 H, m, -$CH_2CH_2CH_2CH_2CH_2COOH$); 1.532-1.644 (6 H, m, -$CH_2CH_2CH_2CH_3$,-$CH_2CH_2CH_2CH_2CH_2COOH$); 2.010 (obscured by AcN signal, -$CH_2CH_2CH_2CH_2CH_2COOH$); 2.240 (2 H, t, J = 7.0 -

$CH_2CH_2CH_2CH_2CH_2COOH$); 3.318 (obscured by MeOH signal, -$CH_2CH_2CH_2CH_3$); 4.791 (2 H, t, J = 7.5, -$CH_2CH_2CH_2CH_2CH_2COOH$); 6.654 (2 H, d, J = 9.0, Phe); 6.918-7.083 (3 H, overlapping d +dd, J = 4.0, thiophene, J = 15.8 and 15.9, -CH=CH-); 7.341 (2 H, d, J = 9.0, Phe); 7.499 (1 H, d, J = 4.0, thiophene); 7.625 (1 H, d, J = 15.6, -CH=); 7.951 (1 H, t, J = 7.3, Phe); 8.126-8.190 (3 H, m, Phe, -CH=, Pyr); 8.280 (1 H, d, J = 8.8 Phe); 8.687 (1 H, d, J = 8.9 Phe); 8.983 (1 H, d, J = 6.7, Pyr). MALDI-TOF *m/z* 581 (M+, 100%), 583 (50), 525 (19).

1-(5-carboxypentyl)-2-[(*E*)-2-(5-{(*E*)-2-[4-(dibutylamino)-phenyl]ethenyl}thiophen-2-yl)ethenyl]-3,3-dimethyl-3*H*-indolium-5-sulfonate (PGH-10)

Intermediates **(2)** and **(12)** (1.18 mmol) produced a blue dye which was isolated by normal phase chromatography eluted with a methanol in dichloromethane gradient. NP-TLC 20% MeOH/CH_2Cl_2 R_f ~ 0.50. Yield 0.87 g (1.09 mmol, 93%). ^{1}H NMR (DMSO-d_6, 300 mHz): 0.936 (6 H, t, J = 7.4, -$CH_2CH_2CH_2CH_3$); 1.283-1.615 (12 H, m, overlapping -$CH_2CH_2CH_2CH_3$, -$CH_2CH_2CH_2CH_2CH_2COOH$); 1.788-1.862 (8 H, m, overlapping -$CH_2CH_2CH_2CH_2CH_2COOH$, Ind-Me_2); 2.229 (2 H, t, J = 7.3, $CH_2CH_2CH_2CH_2CH_2COOH$); 3.342 (obscured by DMSO-d_6 signal, -$CH_2CH_2CH_2CH_3$); 4.540 (2 H, t, J = 6.7, -CH_2CH_3); 6.690 (2 H, d, J = 8.6, Phe), 7.029 (1 H, d, J = 15.2, -CH=); 7.211-7.341 (2 H, dd, J = 16.0 and 7.0, -CH=CH-); 7.396 (1 H, d, J = 4.3, thiophene); 7.495 (2 H, d, J = 9.0, Phe); 7.747-7.822 (2 H, dd, J = 8.3 and 4.8, Ind); 8.023 (1 H, s, Ind); 8.101 (1 H, d, J = 4.0, thiophene); 8.654 (1 H, d, J = 15.8, =CH-). MALDI-TOF *m/z* 677 (M+, 100%), 678 (64), 679 (40).

4-[(*E*)-2-(5-{(*E*)-2-[4-(dibutylamino)phenyl]ethenyl}-thiophen-2-yl)ethenyl]-1-{6-[(2,5-dioxopyrrolidin-1-yl)oxy]-6-oxohexyl}quinolinium bromide (PGH-9-OSu)

N,N,N',N'-Tetramethyl-O-(N-succinimidyl)uronium tetrafluoroborate (TSTU; 275 mg, 0.913 mmol) was added to a solution of PGH-9 dye (0.40 g, 604 mmol) dissolved in dimethylformamide (30 ml) and diisopropylethylamine (300 µl) and stirred for 1 hour at room temperature. Normal phase silica TLC analysis (EtOAc-MeOH, 1:3; R_f = 0.75) showed the quantitative formation of the active NHS ester, which was used directly for the preparation of amino-PEG conjugates.

4-[(*E*)-2-(5-{(*E*)-2-[4-(dibutylamino)phenyl]ethenyl}-thiophen-2-yl)ethenyl]-1-[6-(methoxypolyethylene glycol-750-amino)-6-oxohexyl]quinolinium bromide (PGH-9-PEG750)

$mPEG_{750}$-NH_2 (224 mg 0.300 mmol) was added to previously prepared PGH-9-OSu in DMF/DIPEA solution (14 ml, 0.142 mmol) and mixed for 5 days. Thin layer chromatography [EtOAc-MeOH (1:3)] confirmed product formation by the loss of active ester at the solvent front and increase of PEG product at the origin. The product was isolated by precipitation with 200 ml diethyl ether and collection of the blue solid. Chromatography of this product over alumina eluted with a methanol-dichloromethane gradient caused the dye to change color from deep blue to a light greenish blue. This material was rechromatographed over silica gel, eluting with increasing solvent polarity from dichloromethane, 2-propanol, acetonitrile, methanol, and water. The PEG derivative finally eluted with methylsulfoxide and dimethylformamide followed by 1% acetic acid in toluene-ethyl acetate (1:2). These fractions were concentrated and the residual material was partitioned between ethyl acetate and aqueous potassium chloride (4M). The organic layer was collected and concentrated to an oil, which precipitated upon addition of ether yielding 377 mg of blue solid. MALDI-TOF *m/z* 1211 (98%), 1255 (100), 1299 (92). Mass spectrum contained a series of peaks ranging from 1123-1607 spaced at 44 m/z increments due to polydispersity of PEG group.

Repeat Synthesis (w/o chromatography) $mPEG_{750}$-NH_2 (250 mg 0.333 mmol) was added to previously prepared PGH-9-OSu in DMF/DIPEA solution (0.302 mmol). The reaction was stirred at room temperature until complete as described above. Solvent removed by rotary evaporation using an acetonitrile azeotrope. This product showed sufficient purity by silica and reversed phase TLC (1% acetic acid in methanol-acetonitrile, 1:3) and required no further purification; yield: 540 mg.

4-[(*E*)-2-(5-{(*E*)-2-[4-(dibutylamino)phenyl]ethenyl}-thiophen-2-yl)ethenyl]-1-[6-(methoxypolyethylene glycol-2000-amino)-6-oxohexyl]-quinolinium bromide (PGH-9-PEG2000)

$mPEG_{2000}$-NH_2 (444 mg 0.222 mmol) was added to previously prepared PGH-9-OSu in DMF/DIPEA solution (0.202 mmol) and mixed until reaction was complete (5 days), as described. The reaction was diluted with diethyl ether (30 ml). The blue product was recovered after decanting the solvents and then drying. Chromatography over alumina eluting with a methanol-dichloromethane gradient resulted in a complete change in color from blue to brown-yellow. However, C_2 reversed phase silica eluted with a gradient from 1% acetic acid in water to methanol yielded 130 mg blue solid (0.051 mmol, 23%). MALDI-TOF *m/z* 2531 (M+, 14%), 2575 (13), 2487 (13). Polydispersity of the PEG reagent resulted in a series of product peaks ranging from m/z 2004-3104 with 44 m/z increments.

4-[(*E*)-2-(5-{(*E*)-2-[4-(dibutylamino)phenyl]ethenyl}-thiophen-2-yl)ethenyl]-1-[6-(methoxypolyethylene glycol-5000-amino)-6-oxohexyl]quinolinium bromide (PGH-9-PEG5000)

$mPEG_{5000}$-NH_2 (1.11 g 0.222 mmol) and PGH-9-OSu in DMF/DIPEA solution (0.202 mmol) were mixed until reaction was complete (5 days), as described. Product was purified using C_2 reversed phase silica as described for PGH-9-PEG2000, yielding 104.5 mg (0.0188 mmol, 9%). MALDI-TOF *m/z* 5572 (M+, 89%), 5528 (93), 5616 (84). Polydispersity of the PEG reagent resulted in a series of product peaks ranging from m/z 5044-5968 with 44 m/z increments.

Repeat Synthesis (w/o chromatography) $mPEG_{5000}$-NH_2 (1.66 mg 0.333 mmol) and PGH 9-OSu in DMF/DIPEA solution were reacted as before. Product was isolated by precipitation with ethyl ether (50 ml); centrifuged and solvent decanted, then dried under vacuum yielding 1.2 g (0.215 mmol, 71%).

1,1'-{polyoxyethylene(3,340 MW)-α,ω-diylbis[imino(6-

oxohexane-6,1-diyl)]}bis{4-[(*E*)-2-(5-{(*E*)-2-[4-(dibutylamino)phenyl]ethenyl}thiophen-2-yl)-ethenyl]quinolinium} dibromide (bis-PGH-9-PEG3400)

Polyoxyethylene bisamine (3,350 MW) (372 mg 0.111 mmol) was reacted with PGH-9-OSu and purified using C_2 reversed phase silica, as described for PGH-9-PEG2000, yielding 65 mg (0.0151 mmol, 7.5%). MALDI-TOF *m/z* 4329 (M+, 84%), 4373 (77), 4241 (81). Mass spectrum contained a series of peaks ranging from 3935-4683 spaced at 44 m/z increments due to polydispersity of PEG group.

2-[(*E*)-2-(5-{(*E*)-2-[4-(dibutylamino)phenyl]ethenyl}-thiophen-2-yl)ethenyl]-3,3-dimethyl-1-(6-(methoxypolyethylene glycol-5000-amino)-6-oxohexyl)-3*H*-indolium-5-sulfonate (PGH 10-PEG5000)

TSTU (300 mg, 0.999 mmol) was added to a solution of PGH-10 (0.53 g, 0.666 mmol) in dimethylformamide (39 ml) and diisopropylethylamine (0.4 ml). After 30 min, O-(2-aminoethyl)-O'methoxypolyethylene glycol – 5,000 (3.55 g, 0.710 mmol) was added and the mixture was mixed for 3 days. Product remained at the origin with both C_{18}-RP-TLC (2-propanol-water, 1:1) and silica TLC (methanol-dichloromethane, 1:4). Precipitation and purification of this product by C_2 reversed phase chromatography were as described before, yielding 739.5 mg (0.133 mmol, 20%). λ_{max}(MeOH)/nm 690 (ε/dm^3 mol^{-1} cm^{-1} 119 321). MALDI-TOF *m/z* 5580 (M+, 99%), 5536 (93), 5624 (92). Polydispersity of the PEG reagent gave in a series of product peaks ranging from m/z 5096-6419 with m/z 44 increments.

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