Supporting Information

Fluorescent water-soluble polycationic chitosan polymers as markers for biological 3D imaging

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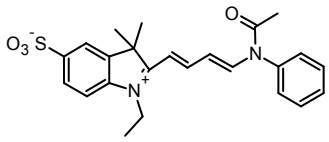
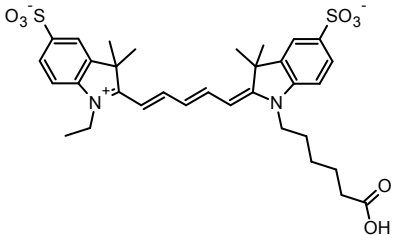
Synthesis, purification, and characterisation of the fluorescent markers



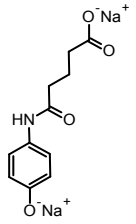
S1

2-[4-(acetylphenylamino)-1,3-butadien-1-yl]-1-ethyl-3,3-dimethyl-5-sulfo-3H-Indolium **(S1)**: In a 1 L 3-necked round bottom flask equipped with a magnetic stirrer and under argon atmosphere, 1-ethyl-2,3,3-trimethylindoleninium-5-sulfonate (10 g, 37.45 mmol, 1 eq) was suspended in acetic anhydride. Malonaldehyde dianylide hydrochloride (10.7 g, 41.2 mmol, 1.1 eq) was added to the flask, followed by acetyl chloride (58.8 g, 749 mmol, 20 eq) and heated at 100 °C for 1.5 hr. The reaction flask was cooled to room temperature and then slowly precipitated in ethyl acetate. The solid was filtered over Gooch funnel and washed with excess ethyl acetate/diethyl ether to yield a greenish-brown solid **S1**, which was directly used for the next step without purification (15.5 g, yield 94%).



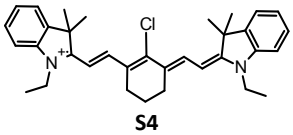
S2

SV645A-01 **(S2)**: In a 1 L flask, **S1** (15.5 g, 35.4 mmol, 1 eq) was suspended in acetic anhydride. 1-(5-carboxypentyl)-2,3,3-trimethylindolium-5-sulfonate (12.5 g, 35.4 mmol, 1 eq) and potassium acetate (9.4 g, 95.5 mmol, 2.7 eq) were added and the reaction was stirred at room temperature for 3 hr. The reaction mixture was slowly precipitated in ethyl acetate. The solid was filtered over Gooch funnel and washed with excess ethyl acetate/diethyl ether. The crude was purified over silica column in methanol and dichloromethane. The column was prepared in 30% methanol and was run from 10 to 100% methanol. Fractions collected were checked using TLC and combined and dried to get pure product. The solvent was dried under vacuum to yield a blue solid **S2** (SV645A-01, 22 g, yield 95%). ^{1}H-NMR (DMSO, 400MHz): δ 1.24 (t, J = 7.2 Hz, 3H), 1.30-1.40 (m, 2H), 1.45-1.57 (m, 2H), 1.67 (s, 12H), 2.12 (t, J = 7.1 Hz, 2H), 4.03-4.17 (m, 4H), 6.29 (dd, J = 13.9, 6.2 Hz, 2H), 6.57 (t, J = 12.4 Hz, 1H), 7.31 (dd, J = 8.3, 2.7 Hz, 2H), 7.62 (ddd, J = 8.2, 4.4, 1.6 Hz, 2H), 7.77 – 7.81 (m, J = 1.9 Hz, 2H), 8.34 (t, J = 13.1 Hz, 2H). ε: 240,000 M-1 cm-1. λab: 648 nm, λem: 667 nm.



S3

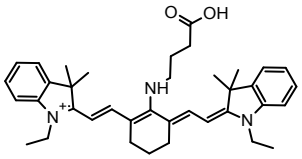
Disodium 4-[(4-hydroxyphenyl)amino]-4-oxobutanoic acid **(S3)**: In a 100 mL round-bottom flask, sodium pellets (1.8 g, 45 mmol, 2 eq) were dissolved completely in 60 mL methanol, and then 4-[(4-hydroxyphenyl)amino]-4-oxobutanoic acid (5 g, 22.5 mmol, 1 eq) was added and stirred altogether at room temperature for 2.5 hr. The reaction was stopped and precipitated in diethyl ether. The precipitate was filtered and dried to obtain a white solid **S3**, which was directly used for the next step without any purification (5 g, 85% yield). The ^{1}H-NMR has been reported in the literature. [1]



S4

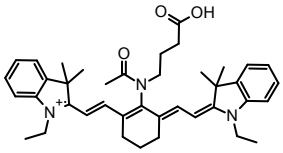
Meso-chloro *N*-ethyl Cyanine 7 **(S4)**: A mixture of 1-ethyl-2,3,3-trimethylindolinium iodide (2 g, 6.34 mmol, 2 eq), Vilsmeier-Haack reagent (1.13 g, 3.17 mmol, 1 eq) and anhydrous sodium acetate (0.93 g, 9.51 mmol, 3 eq) in 20 mL of absolute ethanol was refluxed for 2.5 hr under argon. The reaction mixture was cooled to room temperature, and then concentrated under reduced pressure to yield a brownish green residue. The residue was suspended in DCM, filtered, and dried in vacuum. The crude product was purified by silica gel column chromatography in methanol and dichloromethane (10/90) to yield a golden-green solid **S4** (1.28 g, yield 79.2%).

[1]H-NMR (400 MHz, CDCl3): δ 1.46 (t, 6H), 1.72 (s, 12H), 1.99 (m, 2H), 2.77 (t, 4H), 4.24 (q, 4H), 6.25 (d, J=14 Hz, 2H), 7.25(m, 4H), 7.39 (m, 4H), 8.34 (d, J=14 Hz, 2H). ε: 270,000 M-1 cm -1. λab: 770 nm, λem: 805 nm[2].



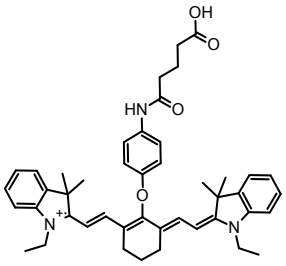
S5

SV620C-01 **(S5)**: A mixture of compound **S4** (0.8 g, 1.25 mmol, 1 eq) and 4-aminobutanoic acid (0.387 g, 3.75 mmol, 3 eq) in DMSO was heated at 65 °C for 4 hr. The reaction mixture was cooled to room temperature and dissolved in DCM. It was extracted twice in water and once in brine. The organic layer was collected, and dried using sodium sulphate, filtered, and dried overnight. The crude product was purified by silica gel column chromatography in methanol and dichloromethane (10/90) to yield a golden-blue solid **S5** (SV620C-01, 0.3 g, yield 42%). [1]H-NMR (400 MHz, DMSO): δ 1.18 (t, 6H), 1.55 (s, 12H), 1.70 (m, 2H), 1.86 (m, 2H), 2.31 (m, 2H), 3.71 (t, 2H), 3.90 (m, 4H), 5.6 (d, J=12 Hz, 2H), 7.01 (m, 4H), 7.23 (t, 2H), 7.37 (d, J=7 Hz, 2H), 7.52 (d, J=12 Hz, 2H). ε: 90,000 M-1 cm -1. λab: 620 nm, λem: 750 nm[2].



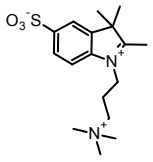
S6

SV770C-01 **(S6)**: In a 2-necked round -bottom flask, compound **S5** (0.05 g, 0.070 mmol, 1 eq) was dissolved in dichloromethane (12 mL) in an ice bath under argon atmosphere. Acetyl chloride (29.25 µL, 0.375 mmol, 5 eq) and N, N-diisopropylethylamine (240.5 µL, 1.875 mmol, 25 eq) were injected into the reaction flask and stirred for 15 mins. The reaction was quenched in 0.1 N HCl solution (20 mL) and then dried off over rotary evaporator. The crude product was purified by silica gel column chromatography in methanol/dichloromethane (10/90) to yield a golden-green solid **S6** (SV770C-01, 0.032 g, yield 75%). [1]H-NMR (400 MHz, DMSO): δ 1.26 (t, 6H), 1.53, 1.61 (s, 12H), 1.82 (m, 2H), 2.36 (m, 2H), 2.65 (m, 2H), 3.29 (s, 2H), 3.66 (t, 2H), 4.19 (m, 4H), 6.23 (d, J=12 Hz, 2H), 7.24 (m, 2H), 7.40 (d, 4H), 7.45 (d, J=7 Hz, 2H), 7.55 (d, J=12 Hz, 2H). ε: 270,000 M-1 cm -1. λab: 790 nm, λem: 805 nm [3].



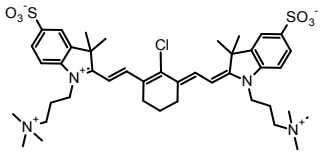
S7

SV770C-02 **(S7)**: A mixture of compound **S4** (0.912 g, 1.42 mmol, 1 eq) and compound **S3** (1.2 g, 4.2 mmol, 3 eq) in DMSO and water (1:1) was heated at 65 °C for 23 hr. The reaction mixture was cooled to room temperature and acidified with 0.1% formic acid. The reaction was extracted in dichloromethane and water, dried over sodium sulfate, and then put on rotary evaporator overnight to give solid crude. The crude product was purified by silica gel chromatography in methanol/dichloromethane (10/90) to yield a golden-green solid compound **S7** (SV770C-02, 0.416 g, yield 42%). [1]H-NMR (400 MHz, DMSO): δ 1.39 (m, 6H) 1.51 (t, 6H), 1.86 (s, 12H), 2.04 (m, 2H), 2.92 (t, J = 5.8 Hz, 4H), 4.45 (q, J = 6.8 Hz, 4H), 6.52 (d, J = 14.2 Hz, 2H), 7.48 (m, 2H), 7.64 (m, 4H), 7.83 (d, J = 7.4 Hz, 2H), 8.46 (d, J = 14.2 Hz, 2H). ε: 270,000 M-1 cm-1. λab: 770 nm, λem: 805 nm.



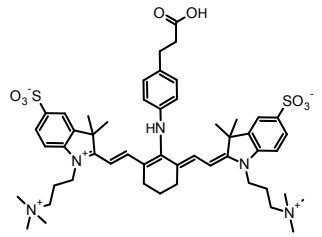
S8

Zwitterionic indolium salt **(S8)**: A mixture of 2,3,3-trimethyl-3H-indole-5-sulfonic acid (1.20 g, 5 mmol, 1 eq) and (3-bromopropyl) trimethyl ammonium bromide (1.56 g, 6.0 mmol, 1.2 eq) in 1,2-dichlorobenzene (16 mL) was heated at 130 °C for 72 hr under argon. The mixture was cooled to room temperature and the solvent was decanted. The crude product was washed with dichloromethane, dissolved in acetone, and re-precipitated into a large volume of ethyl acetate to obtain a red solid **S8** (1.36 g, yield 80%), which was used in the next step without further purification. The [1]H-NMR has been reported in the literature. [4]



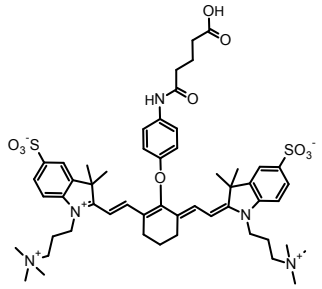
S9

Meso-chloro zwitterionic Cyanine 7 **(S9)**: A mixture of **S8** (0.50 g, 1.48 mmol, 2 eq), Vilsmeier-Haack reagent (0.27 g, 0.73 mmol, 1 eq) and anhydrous sodium acetate (0.25 g, 3.0 mmol, 4 eq) in 10 mL of absolute ethanol was refluxed for 6 hr under argon. The reaction mixture was cooled to room temperature, and then concentrated under reduced pressure to yield a brownish green residue. The crude product was washed with dichloromethane. The residue was suspended in methanol/ dichloromethane (25/75), filtered, and dried in vacuum to yield a golden-green solid **S9** (0.5 g, yield 84.9%). ^{1}H-NMR (400 MHz, D2O): δ 1.72 (s, 12H), 1.88 (m, 2H), 2.18 (m, 4H), 2.74 (m, 4H), 3.08 (s, 18H), 3.49 (m, 4H), 4.18 (m, 4H) m 6.37 (d, J=15 Hz, 2H), 7.45 (d, J=6 Hz, 2H), 7.69 (d, J=6 Hz, 2H) 7.85 (s, 2H), 8.31 (d, J=15 Hz, 2H). ε: 270,000 M-1 cm -1. λab: 770 nm, λem: 805 nm. The characterisation of the compound corresponds to the data reported in the literature[5].



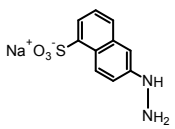
S10

SV700Z-01 **(S10)**: A mixture of compound **S9** (1 g, 1.18 mmol, 1 eq) and 3-(4-aminophenyl) propanoic acid (0.78 g, 4.73 mmol, 4 eq) in DMSO was heated at 65 °C overnight. The reaction mixture was cooled to room temperature and precipitated in dichloromethane. The crude product was purified by reverse-phase C18 chromatography to yield a blue solid **S10** (SV700Z-01, 0.6 g, yield 55%). ^{1}H-NMR (400 MHz, D2O): δ 1.26 (s, 12H), 1.75 (m, 2H), 2.17(m, 4H), 2.38 (m, 2H), 2.51 (m, 3H), 2.80 (m, 2H), 3.14 (s, 18H), 3.48 (m, 4H), 4.01 (s, 4H), 5.98 (d, J= 18 Hz, 2H), 7.11 (m, 6H), 7.76 (m, 4H), 7.99 (d, J=15 Hz, 2H). ε: 76,000 M-1 cm-1. λab: 706 nm, λem: 790 nm. The characterisation of the compound corresponds to the data reported in the literature[5].



S11

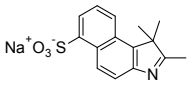
SV770Z-01 **(S11)**: A mixture of compound **S9** (0.5 g, 0.47 mmol, 1 eq) and compound **S3** (0.5 g, 1.88 mmol, 4 eq) in DMSO and water (1:1) was heated at 65 °C for 2.5 hr. The reaction mixture was cooled to room temperature and precipitated slowly in ethyl acetate and absolute ethanol (1:1) with 0.1% formic acid. The precipitate was filtered and washed with excess ethanol. The crude product was purified by reverse-phase C18 chromatography to yield a golden-green solid compound **S11** (SV770Z-01, 0.205 g, yield 42%). %). ^{1}H-NMR (400 MHz, D2O): δ 1.15 (s, 12H), 1.9 (m, 4H), 2.25 (m, 6H), 2.4 (t, 2H), 2.63 (m, 4H), 3.13 (s, 18H), 3.49 (m, 4H), 4.04 (s, 4H), 6.14 (d, J= 14 Hz, 2H), 6.96 (d, J= 8 Hz, 2H), 7.17 (d, J= 8 Hz, 2H), 7.40 (d, J= 8 Hz, 2H), 7.68 (s, 1H), 7.72 – 7.86 (m, J = 12.0 Hz, 4H). ε: 270,000 M-1 cm-1. λab: 775 nm, λem: 805 nm.



S12

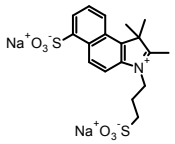
6-Hydrazinyl-1-naphthalenesulfonic acid **(S12)**: 5-amino-2-naphthalenesulfonic acid (60 g, 268.8 mmol, 1 eq) was added to a 1 L jacketed flask connected to a cryostat at -5°C. Concentrated hydrochloric acid (300 mL) was added very slowly with continuous stirring. Once fully dissolved, a solution of sodium nitrate (18.55 g, 268.8 mmol, 1 eq) in 120 mL water was added very slowly using a dropping funnel over 60 mins. The reaction was run for 30 mins at 2 °C. Temperature was again brought down to -5 °C and a solution of tin (II) chloride dihydrate (182 g, 806.4 mmol, 3 eq) in concentrated hydrochloric acid (180 mL) was added slowly using a dropping

funnel over 2 hr. The reaction was allowed to run at 2 °C for 1 hr. The reaction mixture was filtered and washed with excess absolute ethanol. The precipitate was dried under vacuum for 2 days to give a yellow solid **S12** (75 g, yield 100 %). The NMR showed impurities alongside the product peaks. This was used directly for the next step without purification.



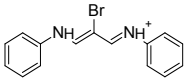
S13

1,1,2-Trimethyl-*1H*-benz[e]indole-6-sulfonic acid **(S13)**: Compound **S12** (75 g, 288 mmol, 1 eq) was taken in a round bottom flask with 3-methyl-2-butanone (74.5 g, 865 mmol, 3 eq) and 150 mL of acetic acid. The reaction mixture was refluxed at 130 °C for 5 hr with continuous stirring. The reaction was then cooled down and precipitated into ethyl acetate. The precipitate was filtered over Gooch funnel and dried under vacuum to give compound **S13** (44 g, yield 50%). The NMR showed impurities alongside the product peaks. This was used directly for the next step without purification.



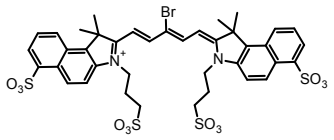
S14

1,1,2-trimethyl-6-sulfo-3-(3-sulfopropyl)-*1H*-Benz[e]indolium **(S14)**: Compound **S13** (1.5 g, 4.8 mmol, 1 eq) was taken in a round-bottom flask and dissolved completely in sulfolane (5 mL), to which 1,3-propanesultone (0.631 mL, 7.2 mmol, 1.5 eq) was added. Altogether they were refluxed at 130 °C for 20 hr. The reaction was then cooled down and the solvent was decanted. 5 mL of methanol was added to quench the reaction and dissolve the solid block, which was then precipitated in acetone. The precipitate was filtered over Gooch funnel and washed with acetone, ethanol, and diethyl ether. The solid was dried under vacuum to give compound **S14** (1.45 g, yield 66.6%). The NMR showed impurities alongside the product peaks, but the solid was used in the following reaction without further purification.



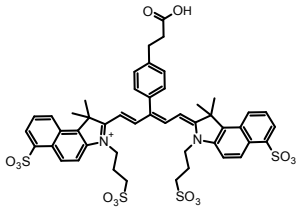
S15

N-[(*1E,2Z*)-2-bromo-3-(phenylamino)prop-2-en-1-ylidene]anilinium **(S15)**: In a 250 mL vacuum Erlenmeyer flask, aniline (7.08 g, 76.02 mmol, 2 eq), was dissolved in 30 mL absolute ethanol. Separately, mucobromic acid (10 g, 38.01 mmol, 1 eq) was dissolved in 30 mL absolute ethanol and transferred to a closed dropping funnel. The solution was added dropwise to the aniline mixture with constant cooling. The vacuum flask was equipped with a silicon tube that was put in a beaker containing ethanol to control the carbon dioxide formation. At the end of the addition, the mixture was transferred to a 250 mL beaker equipped with magnetic stirrer and was heated ion a water bath till its volume reduced to half. The resulting solution was cooled down and chilled in the freezer for 1 hr. The yellow crystalline precipitate was filtered and washed with cold ethanol and ether to obtain a yellow solid **S15** (6.4 g, yield 45.5%). The compound has been previously reported in literature. [6]



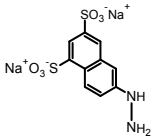
S16

Meso-bromo tetra-sulfonate Cyanine 5.5 **(S16)**: In a 100 mL round-bottom flask, Compound **S14** (1 g, 2.4 mmol, 2 eq) was stirred along with triethylamine (3.34 mL, 24 mmol, 10 eq) in absolute ethanol (10 mL) at room temperature for 30 mins till it fully dissolved. Compound **S15** (0.458 g, 1.2 mmol, 1 eq) and acetic anhydride (5 mL) was added to the flask and the reaction was stirred at 80 °C for 1 hr. The reaction was cooled and precipitated in excess acetone. The precipitate was filtered over Gooch funnel and was dried under vacuum. The crude was purified using reverse-phase C18 column chromatography to yield a blue powder **S16** (0.56 g, yield 50 %). There was still some triethylamine present in the sample, which was purified in the later steps. ^{1}H-NMR (400 MHz, D2O): δ 1.54 (s, 12H), 1.93-2.24 (m, 4H), 2.60-3.18 (m, 4H), 3.94-4.28 (m, 4H), 6.00 (d, J = 14.0 Hz, 2H), 7.14-8.25 (m, 10H), 8.72 (d, J = 14.0 Hz, 2H). ε: 240,000 M-1 cm-1. λab: 674 nm, λem: 703 nm.



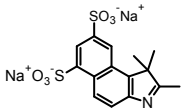
S17

SV680A-03 **(S17)**: In a 10 mL round-bottom flask under argon atmosphere, Compound **S16** (0.075 g, 0.08 mmol, 1 eq) was taken along 4-(2-carboxyethyl) benzene boronic acid (0.046 g, 0.24 mmol, 3 eq) and cesium carbonate (0.052 g, 0.16 mmol, 2 eq) in absolute ethanol and water (50/50) and stirred at room temperature for 30 mins, till it was fully dissolved. Tetrakis(triphenylphosphine)palladium (0) (0.018 g, 0.016 mmol, 20% by weight) was added to the flask and the temperature was raised to 80 °C. The reaction was stirred for 4 hr and then extracted in ethyl acetate and water. The crude was purified using reverse-phase C18 column chromatography to yield a golden-blue solid **S17** (SV680A-03, 0.05 g, yield 63 %). %). ^{1}H-NMR (400 MHz, D2O): δ 1.70 (s, 12H), 1.82-1.97 (m, 4H), 2.45 (t, 2H), 2.56-2.68 (m, 4H), 2.86 (t, 2H), 3.64-3.86 (m, 4H), 5.48 (d, J = 13.9 Hz, 2H), 6.93 (d, J = 7.0 Hz, 2H), 7.31 (d, J = 7.7 Hz, 2H), 7.42 (t, J = 7.6 Hz, 2H), 7.49 (d, J = 9.2 Hz, 1H), 7.86 (d, J = 7.0 Hz, 2H), 8.06 (d, J = 14.2 Hz, 2H), 8.22 (d, J = 7.9 Hz, 2H), 8.61 (d, J = 9.4 Hz, 2H). ε: 240,000 M-1 cm-1. λab: 674 nm, λem: 703 nm.



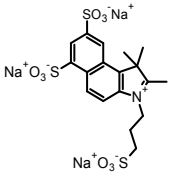
S18

6-Hydrazinyl-1,3-naphthalenedisulfonic acid **(S18)**: 6-amino-1,3-naphthalenedisulfonic acid disodium salt (31.1 g, 89.58 mmol, 1 eq) was added to a 1 L jacketed flask connected to a cryostat at -5°C. Concentrated hydrochloric acid (100 mL) was added very slowly with continuous stirring. Once fully dissolved, a solution of sodium nitrate (9.27 g, 89.58 mmol, 1 eq) in 40 mL water was added very slowly using a dropping funnel over 60 mins. The reaction was run for 30 mins at 2 °C. Temperature was again brought down to -5 °C and a solution of tin (II) chloride dihydrate (60.62 g, 268.74 mmol, 3 eq) in concentrated hydrochloric acid (60 mL) was added slowly using a dropping funnel over 2 hr. The reaction was allowed to run at 2 °C for 1 hr. The reaction mixture was filtered and washed with excess absolute ethanol. The precipitate was dried under vacuum for 2 days to give a yellow solid **S18** (16 g, yield 50 %), which was used directly for the next step without purification. The compound was impure but was used directly for the next step, as reported in the literature[7].



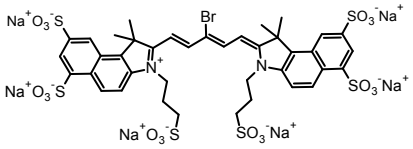
S19

1,1,2-Trimethyl-*1H*-benz[e]indole-6,8-disulfonic acid **(S19)**: Compound **S18** (7 g, 19.3 mmol, 1 eq) was taken in a round bottom flask with 3-methyl-2-butanone (6.125 mL, 57 mmol, 3 eq) and 25 mL of acetic acid. The reaction mixture was refluxed at 130 °C for 5 hr with continuous stirring. The reaction was then cooled down and precipitated into ethyl acetate. The precipitate was filtered over Gooch funnel and dried under vacuum to give compound **S19** (6 g, yield 75%), which was used directly for the next step without purification. The compound was impure but was used directly for the next step, as reported in the literature[7].



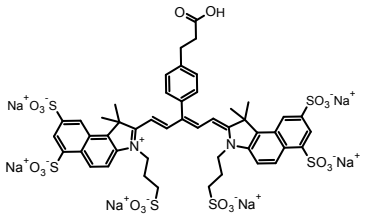
S20

1,1,2-trimethyl-6,8-disulfo-3-(3-sulfopropyl)-1H-Benz[e]indolium **(S20)**: Compound **S19** (6.5 g, 14.5 mmol, 1 eq) was taken in a round-bottom flask along with 1,3-propanesultone (2 mL, 21.8 mmol, 1.5 eq) in 1,2-dichlorobenzene (35 mL) and refluxed at 130 °C for 20 hr. The reaction was then cooled down and the solvent was decanted. The precipitate was triturated with 40 mL of ethyl acetate and filtered over Gooch funnel. The solid precipitate was further triturated with ethyl acetate (4 X 40 mL) after which it was re-dissolved in hot methanol (100 mL) and precipitated in isopropanol. The precipitate was filtered and washed with isopropanol, diethyl ether and ethyl acetate. The solid was dried under vacuum to give compound **S20** (3.5 g, yield 43.4%), which was used directly for the next step without purification. The compound was impure but was used directly for the next step, as reported in the literature[7].



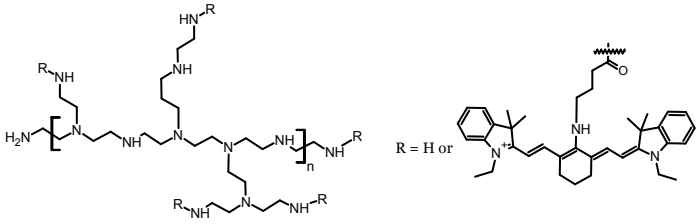
S21

Meso-bromo hexa-sulfonate Cyanine 5.5 **(S21)**: In a 100 mL round-bottom flask, Compound **S20** (3.5 g, 6.3 mmol, 2 eq) was stirred along with triethylamine (8.78 mL, 63 mmol, 10 eq) in absolute ethanol (30 mL) at room temperature for 30 mins till it fully dissolved. Compound **S15** (1.202 g, 3.15 mmol, 1 eq) and acetic anhydride (10 mL) was added to the flask and the reaction was stirred at 80 °C for 2 hr. The reaction was cooled and precipitated in excess acetone. The precipitate was filtered over Gooch funnel and was dried under vacuum. The crude was purified using reverse-phase C18 column chromatography to yield a blue powder **S21** (1.72 g, yield 50 %). ^{1}H-NMR (400 MHz, D2O): δ 1.81 (s, 12H), 2.16-2.28 (m, 4H), 2.93-3 (m, 4H), 4.36 (t, 4H), 6.33 (d, J = 13.4 Hz, 2H), 7.83 (d, J = 9.4 Hz, 2H), 8.18 (dd, J = 15.2, 7.5 Hz, 4H), 8.64 (d, 2H), 8.77 (d, J = 9.8 Hz, 2H). ε: 240,000 M-1 cm-1. λab: 674 nm, λem: 703 nm. The characterisation of the compound corresponds to the data reported in the literature[7].



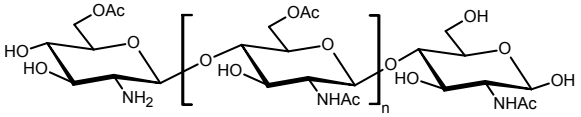
S22

SV680A-02 **(S22)**: In a 10 mL round-bottom flask under argon atmosphere, Compound **S21** (0.09 g, 0.057 mmol, 1 eq) was taken along 4-(2-carboxyethyl) benzene boronic acid (0.033 g, 0.173 mmol, 3 eq) and cesium carbonate (0.037 g, 0.115 mmol, 2 eq) in absolute ethanol and water (50/50) and stirred at room temperature for 30 mins, till it was fully dissolved. Tetrakis (triphenylphosphine) palladium (0) (0.013 g, 0.0115 mmol, 20% by weight) was added to the flask and the temperature was raised to 80 °C. The reaction was stirred for 4 hr and then extracted in ethyl acetate and water. The crude was purified using reverse-phase C18 column chromatography to yield a golden-blue solid **S22** (SV680A-02, 0.05 g, yield 80 %). ^{1}H-NMR (400 MHz, D2O): δ 1.93 (s, 12H), 1.99-2.07 (m, 4H), 2.53 (t, J = 7.8 Hz, 2H), 2.66-2.78 (m, J = 7.7 Hz, 4H), 2.93 (t, J = 7.8 Hz, 2H), 3.92-4.04 (m, 4H), 5.76 (d, J = 14.1 Hz, 2H), 7.24 (d, J = 8.1 Hz, 2H), 7.47 (d, J = 7.7 Hz, 2H), 7.70 (d, J = 9.2 Hz, 2H), 8.32 – 8.21 (m, J = 9.2 Hz, 4H), 8.79 – 8.65 (m, J = 5.8 Hz, 4H). ε: 240,000 M-1 cm-1. λab: 674 nm, λem: 703 nm. The characterisation of the compound corresponds to the data reported in the literature[7].



S23

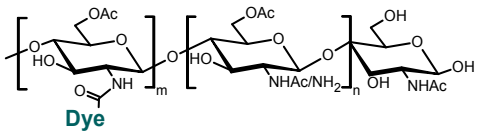
SV620C-01-PEI **(S23)**: In a 25 mL round-bottom flask, a mixture of SV620C-01 **S5** (0.025 g, 0.04 mmol, 15 eq), N-hydroxy succinimide (0.02 g, 0.18 mmol, 60 eq) and 1-ethyl-3-(3-dimethylamino- propyl) carbodiimide (0.028 g, 0.18 mmol, 60 eq) was stirred in 5 mL anhydrous DMSO at room temperature under argon for 20 mins. To this reaction mixture, a solution of polyethyleneimine (PEI) 70 kDa in size (0.25 g, 0.003 mmol, 1 eq) in 10 mL anhydrous DMSO was added and stirred altogether for 4.5 hr at room temperature. The reaction was stopped, and the content of the flask was transferred to a dialysis bag. The reaction mix was dialysed against 1X PBS buffer (pH 7.4) for 36 hr with change of the dialysis medium every 12 hours. The resulting solution inside the dialysis bag was lyophilised to obtain a blue powder **S23** (SV620C-01-PEI, 0.17 g, 70% yield). ε: 90,000 M-1 cm -1. λab: 620 nm, λem: 750 nm.



S24

WS Chitosan **(S24)**: In a 100 mL round-bottom flask, Chitosan (2.2 g, 0.0169 mmol, 1 eq) was stirred in 22 mL of methane sulfonic acid. After 1.5 hr, acetyl chloride was added to this flask, and was stirred altogether for 5 hr. The reaction was stopped by adding 50 g ice to the flask and the reaction mixture was transferred to a dialysis bag and dialysed against Milli-Q water. After 36 hours, the content of the dialysis bag was neutralised using sodium bicarbonate solution and continued to dialyse for 2 days. The dialysing

medium was changed every 12 hours. After 2 days, the content of the dialysis bag was freeze-dried to give a white solid **S24** (WS Chitosan, 1.45 g, yield 66.4%). ^{1}H-NMR (600 MHz, 0.5 M DCl/D2O): 1.58-2.0 (m, 4.46 H), 2.52 (s, 0.27 H), 3.22-3.70 (m, 8.36 H), 4.58 (m, 0.79H). The characterisation of the compound corresponds to the data reported in the literature[8].

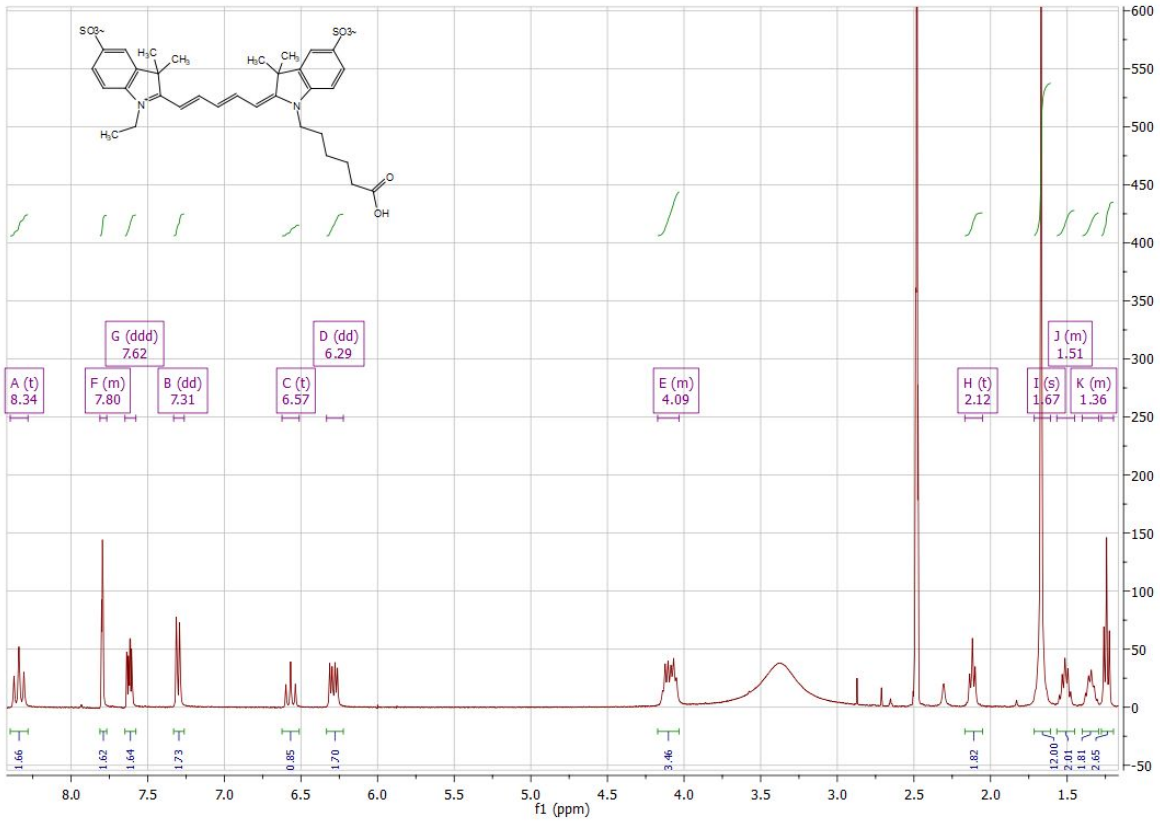


where m is between 1 to 25 for all the kidney imaging markers

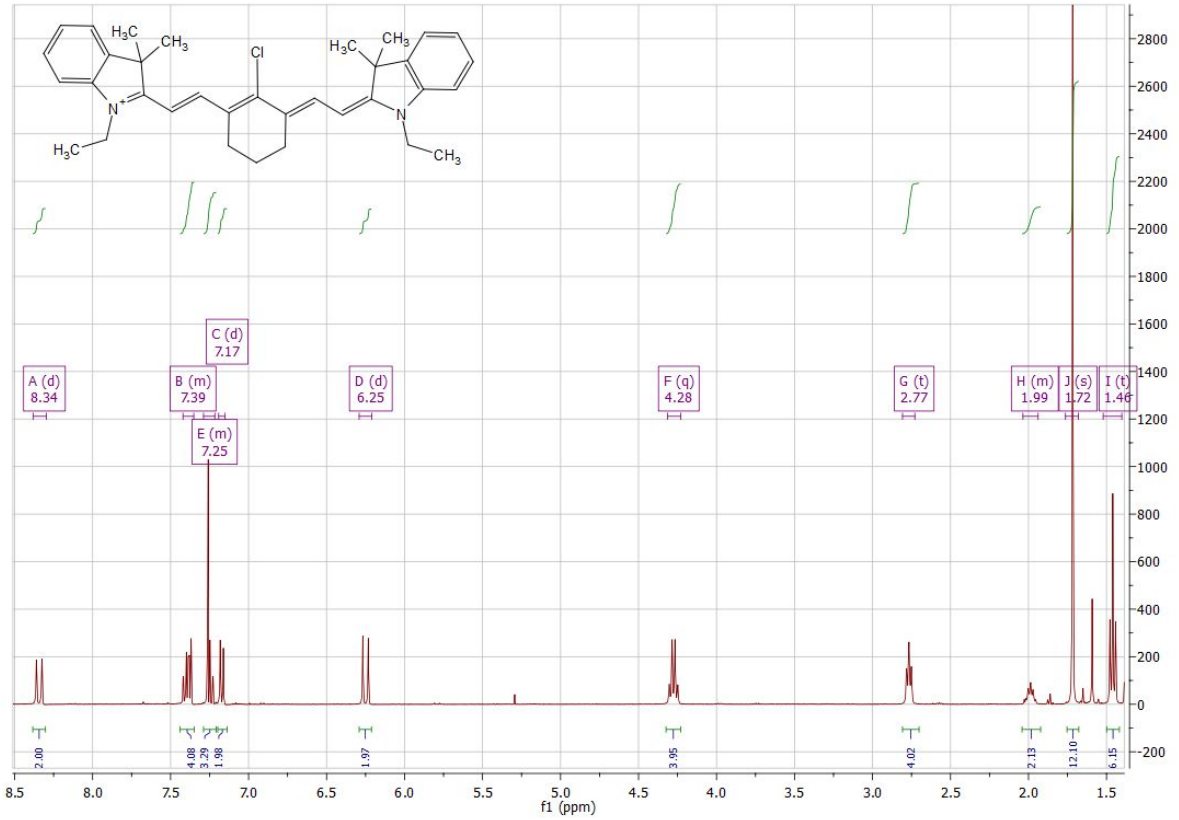
S25

Fluorescent WS Chitosan: In a 25 mL round-bottom flask, a mixture of fluorescent dye (SV620C-01 **(S5)**/ SV770C-01**(S6)**/SV700Z-01**(S10)**/SV770C-02**(S7)**/SV770Z-01**(S11)**/SV645A-01**(S2)**/SV680A-03**(S17)**/SV680A-02 **(S22)**, 40 eq), N-hydroxy succinimide (200 eq) and 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (200 eq) was stirred in anhydrous DMSO at room temperature under argon for 20 mins. To this reaction mixture, a solution of WS Chitosan **S24** (1 eq) in anhydrous DMSO was added and stirred altogether for 4.5 hr at room temperature. The reaction was stopped, and the content of the flask was transferred to a dialysis bag. The reaction mix was dialysed against 1X PBS buffer (pH 7.4) for 24 hr with change of the dialysis medium every 12 hours, and later against Milli-Q water for another 12 hours. The resulting solution inside the dialysis bag was lyophilised to obtain a lyophilised powder with varying optical properties, depending on the dye used for conjugation.

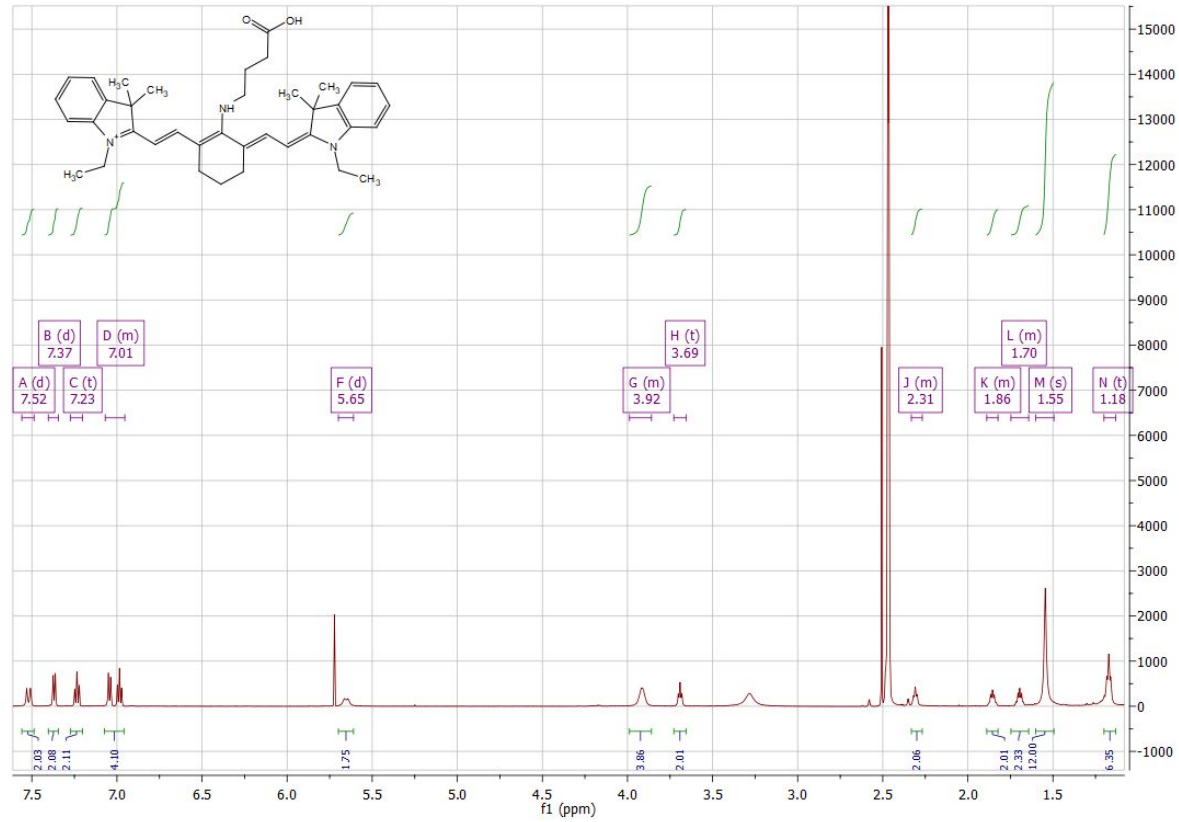
<u>Supporting characterisation images:</u>



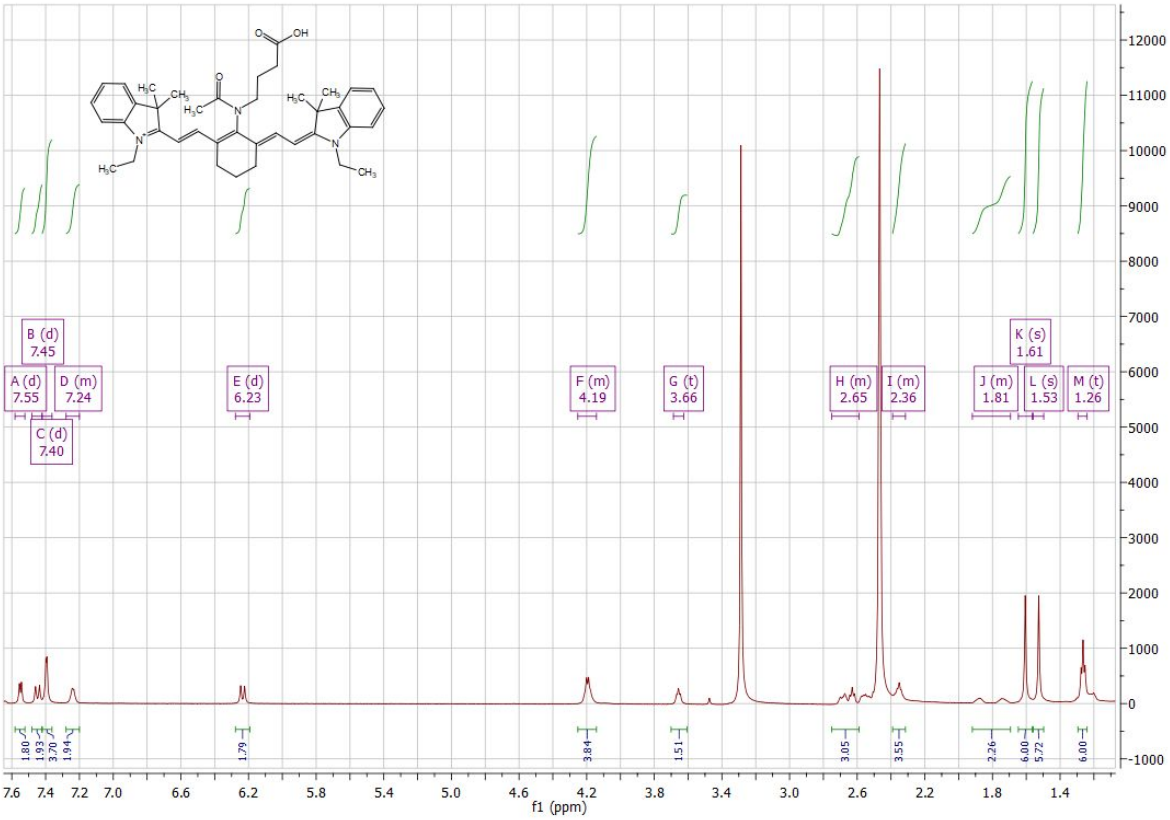
S26 ^{1}H-NMR of SV645A-01 **(S2)**



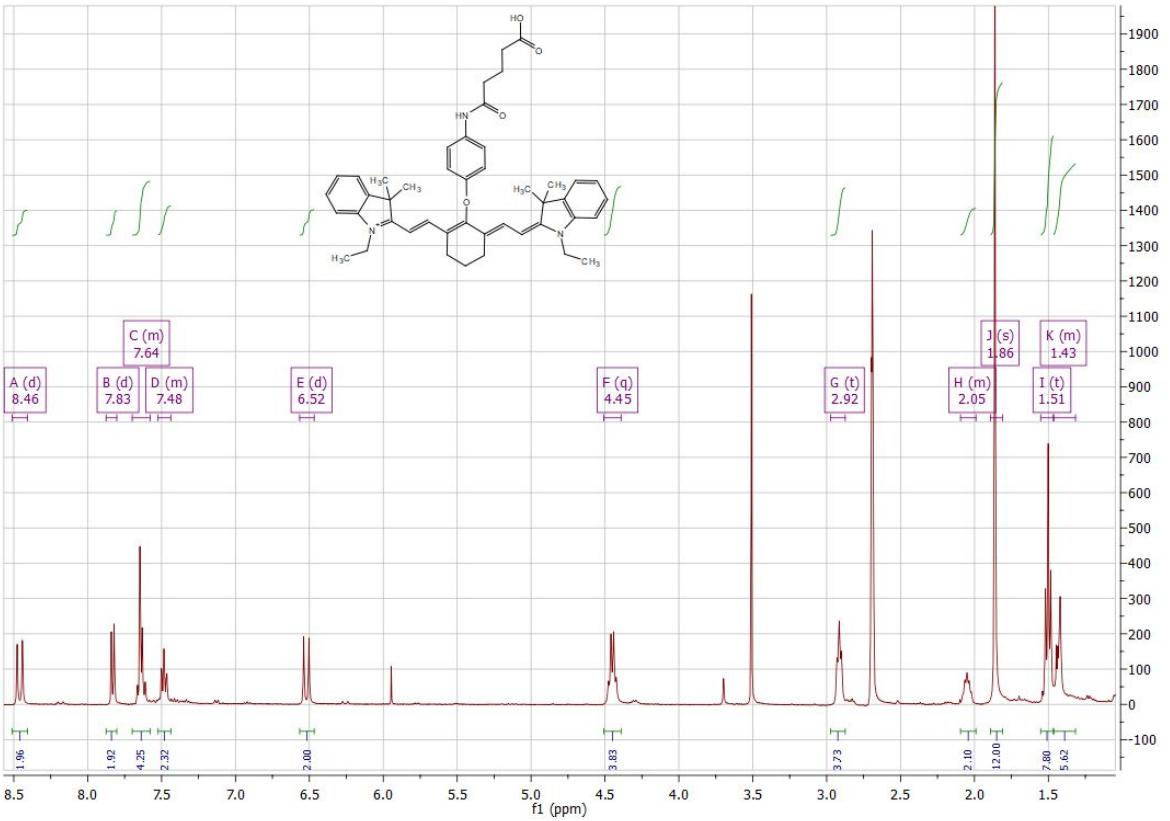
S27 ^{1}H-NMR of Meso-chloro N-ethyl Cyanine 7 **(S4)**



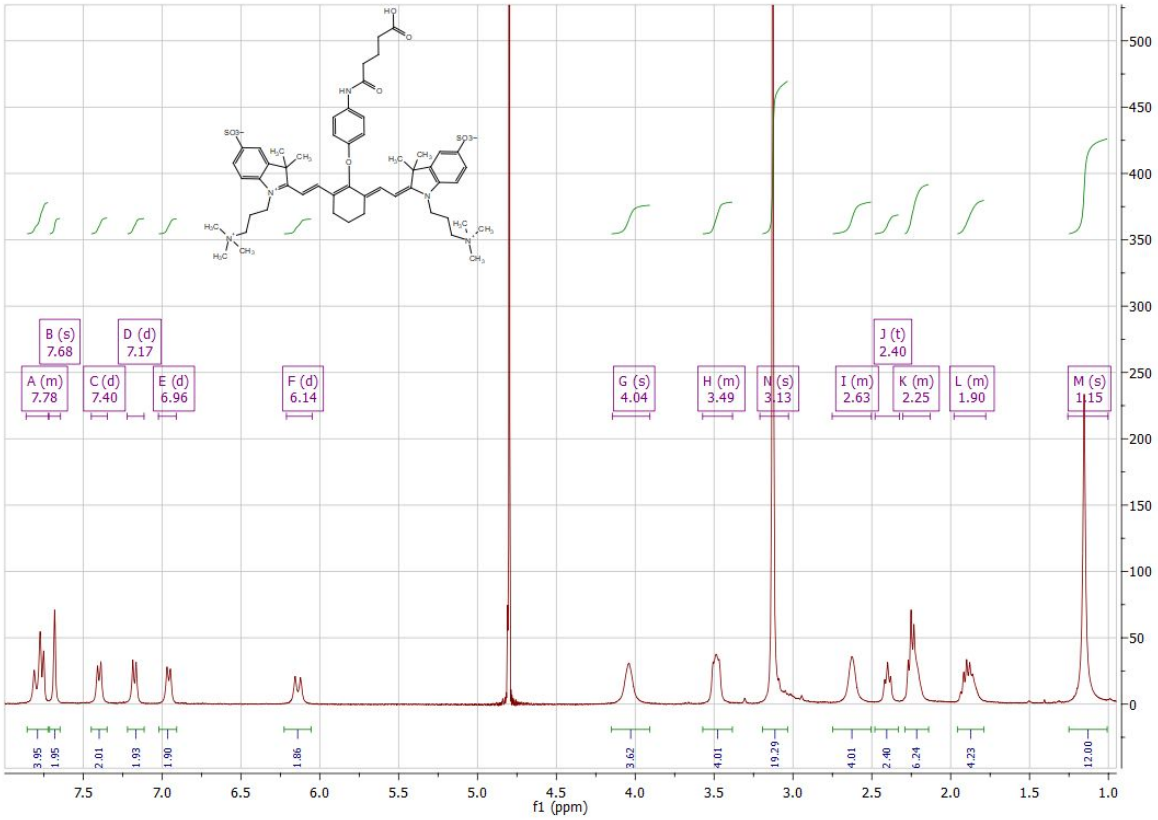
S28 ^{1}H-NMR of SV620C-01 **(S5)**



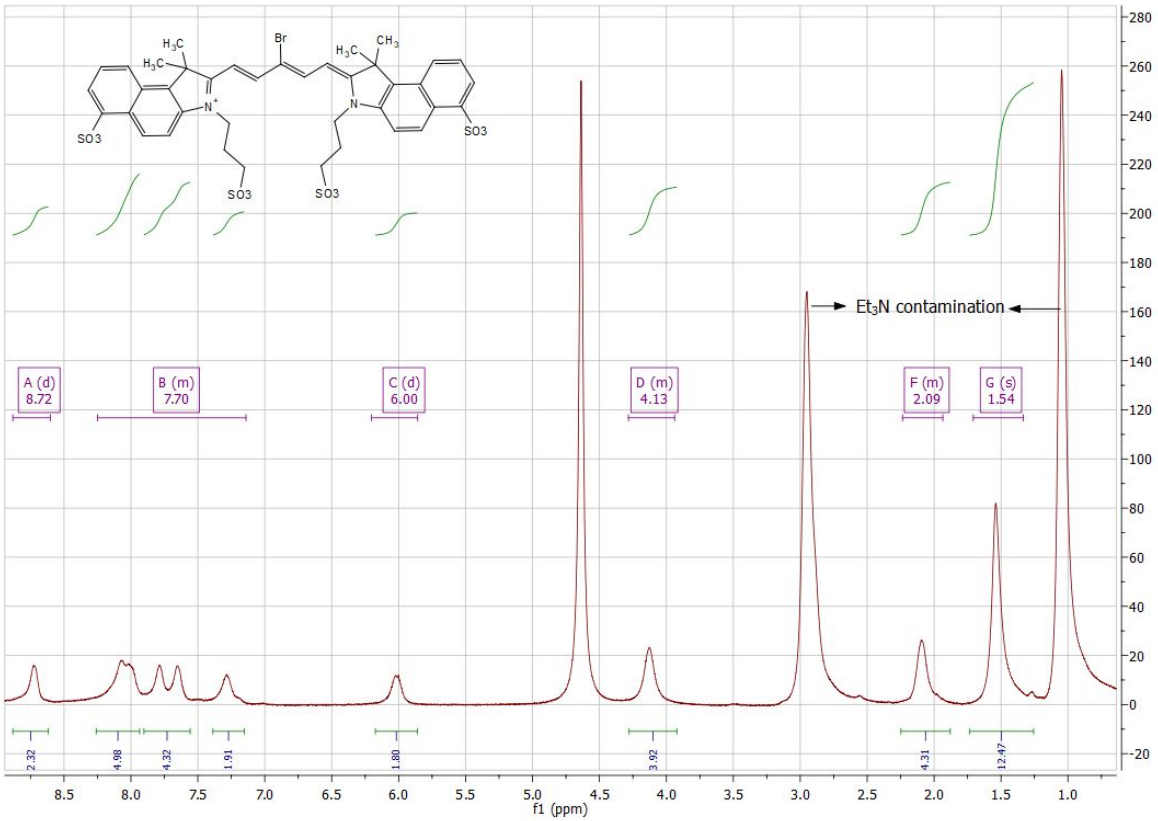
S29 ^{1}H-NMR of SV770C-01 **(S6)**



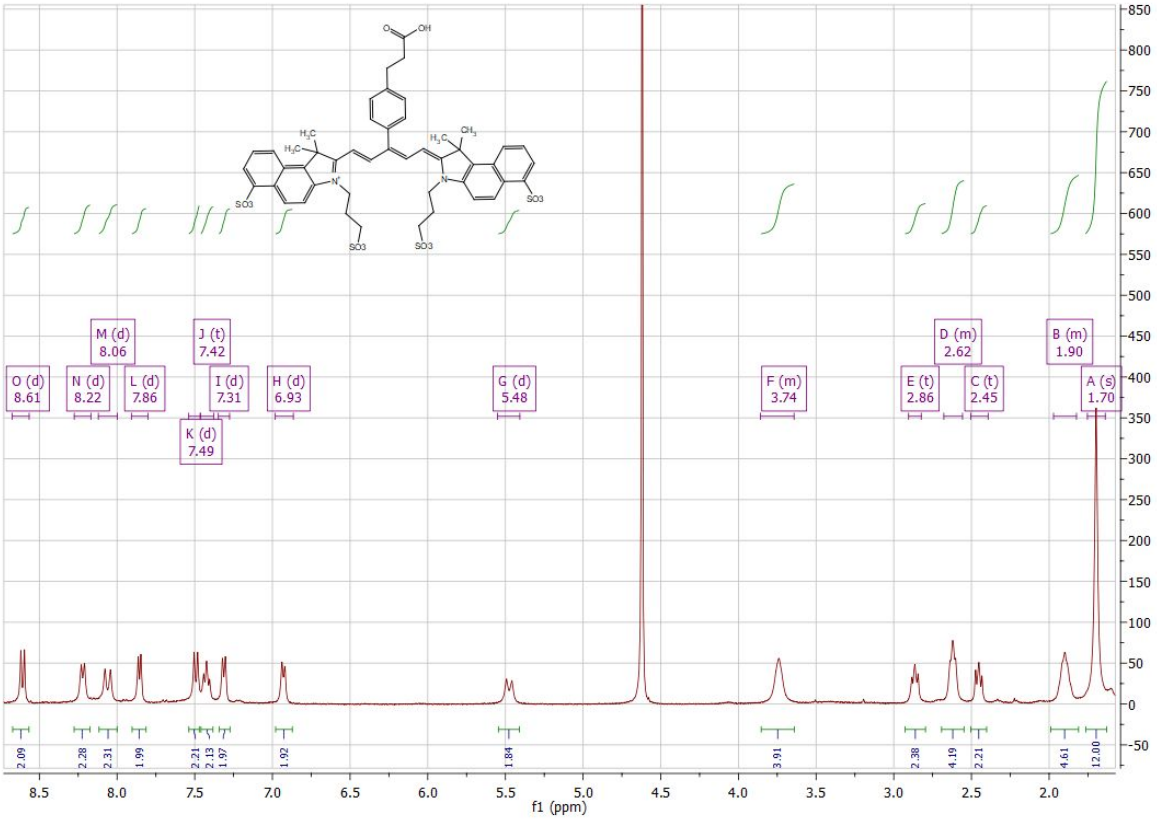
S30 ^{1}H-NMR of SV770C-02 **(S7)**



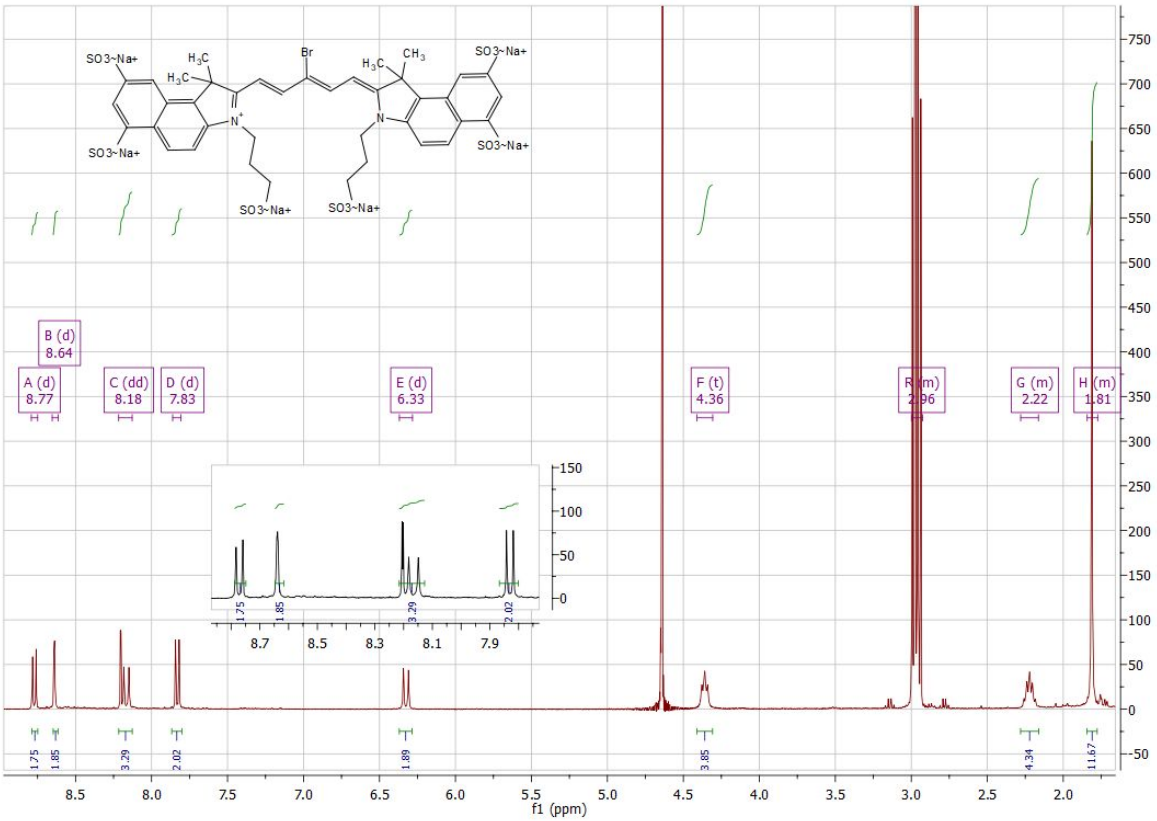
S31 ^{1}H-NMR of SV770Z-01 **(S11)**



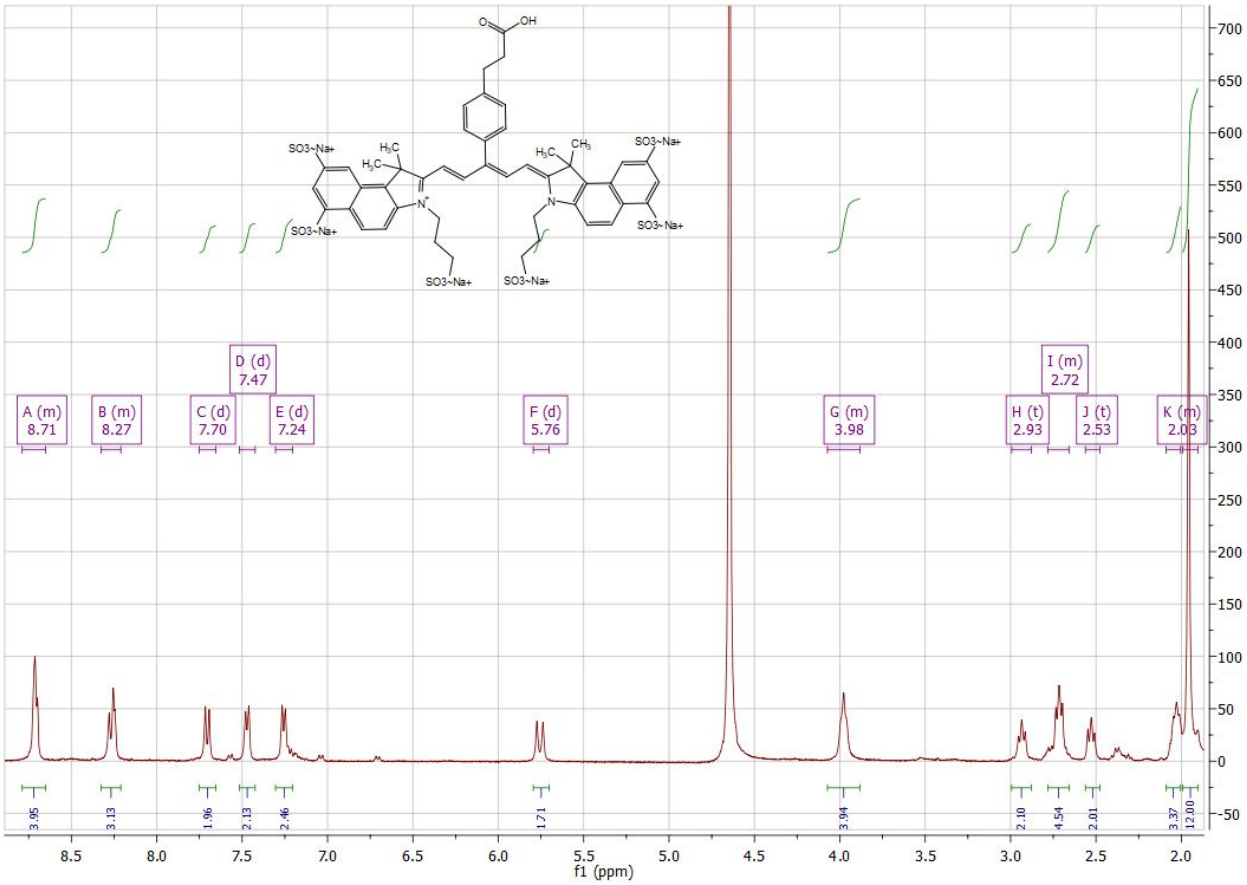
S32 ^{1}H-NMR of Meso-bromo tetra-sulfonate Cyanine 5.5 **(S16)**



S33 ^{1}H-NMR of SV680A-03 **(S17)**



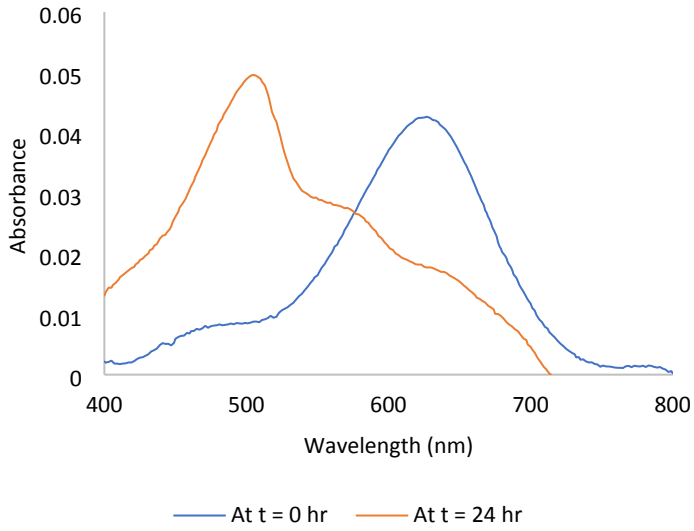
S34 ^{1}H-NMR of Meso-bromo hexa-sulfonate Cyanine 5.5 **(S21)**



S35 ^{1}H-NMR of SV680A-02 **(S22)**

Proof of electrostatic interactions between MH1148 and PEI

In a 20 mL beaker, a mixture of MHI-148 (0.010 g, 0.0141 mmol, 3eq) was stirred with a solution of polyethyleneimine (PEI) 70 kDa in size (0.335 g, 0.004 mmol, 1 eq) in 10 mL anhydrous DMSO at room temperature for 20 mins. The content of the beaker was transferred to a dialysis bag. Absorbance of this mixture was recorded using CARY-Bio-100 Spectrophotometer. The mixture was then dialysed against 1X PBS buffer (pH 7.4) for 24 hr with change of the dialysis medium every 12 hours. Absorbance of the mixture was recorded again after 24 hours to confirm the staining of the polymer by the dye via electrostatic interactions.



S36 Absorption spectra of a mixture of MHI-148 and PEI before dialysis at t = 0 hr (in blue) and after dialysis at the end of 24 hr (in orange). The peak at 465 nm comes from the partially degraded dye which increases in intensity over time.

Photostability characterization of NIR Dyes

The spectroscopic characterization and the photostability analyses were performed in phosphate buffered saline (PBS) at pH 7.4, 25 °C, using a spectrophotometer (Cary 4000 UV-Vis, Agilent) and a spectrofluorometer (FS5, Edinburgh Instruments), respectively, at the Department of Medicine and Surgery, University of Parma.

Four of the investigated dyes (SV620C-01, SV680A-02, SV700Z-01, and SV770Z-01) were conjugated with WS Chitosan, and SV620C-01 was also conjugated with PEI for comparative analysis.

The NIR dyes were subjected to continuous illumination by keeping the excitation source open throughout the entire duration of the analysis over a total duration of 150 minutes to monitor the changes in fluorescence intensity over time. SV620C-01 (conjugated with WS Chitosan and PEI) was excited at 622 nm and fluorescence emission was monitored at 750 nm, while SV680A-02, SV700Z-01 and SV770Z-01 (conjugated only with WS Chitosan) were excited at 677, 706 and 770 nm and fluorescence emission were monitored at 703, 790 and 790 nm, respectively. Absorption and fluorescence emission spectra were acquired just before starting the continuous illumination (initial time point) and after 150 minutes of illumination (end of the photostability assay).

Biological sample preparation for OTC

All the tissue samples for OTC were collected after animal perfusion with 10 mL of SV680A-02-WS Chitosan and SV770Z-01-WS Chitosan solution, administrated at the concentration of 2 μM for the retrograde perfusion and with 10 mL of the PEI conjugates at the concentration of 14.2 μM. The samples were subjected to an optimised clearing procedure based on the use of ECi (Ethyl Cinnamate, SigmaAldrich, Germany, STBH5252, R.I. 1.558 at 25°C) [2]. The procedure requires the use of an automated tissue processor (TP1020, Leica Biosystem, Nußloch, Germany), including four baskets filled with increasing concentrations of ethanol (50%, 80%, 99%, 99%) for the dehydration steps (45 minutes each) prior to ECi clearing that occurs for 2 hours and can be extended overnight.
The cleared tissues were mounted on a petri dish (Thermo Fisher Scientific, S33580A) and immersed in immersion oil (RI = 1.51, Merck, Cat. No.1046990100) or ECi (SigmaAldrich, Germany, STBH5252, R.I. 1.558 at 25°C) for the imaging. The imaging was performed using a confocal microscope TCS SP8 and STELLARIS 8 (Leica Microsystems GmbH, Mannheim, Germany) equipped with a long-working distance objective (HC FLUOTAR 16x/0.60 IMM objective, Leica Microsystems, Germany) ensuring in-depth imaging of thick specimens. MHI-148-PEI, SV620C-01-PEI and SV680A-02-WS Chitosan were excited at 638 nm and fluorescence was detected using a Cy7 and Alexa 680 emission filter, while SV770Z-01-WS Chitosan was imaged by Stellaris 8. 3D reconstructions were rendered using Leica LAS X software and are based on the adjustments made in minimum and maximum intensity and opacity of the 3D viewer section in the LAS X software. All the data acquired were saved as live files and read into LAS X or Fiji (ImageJ).

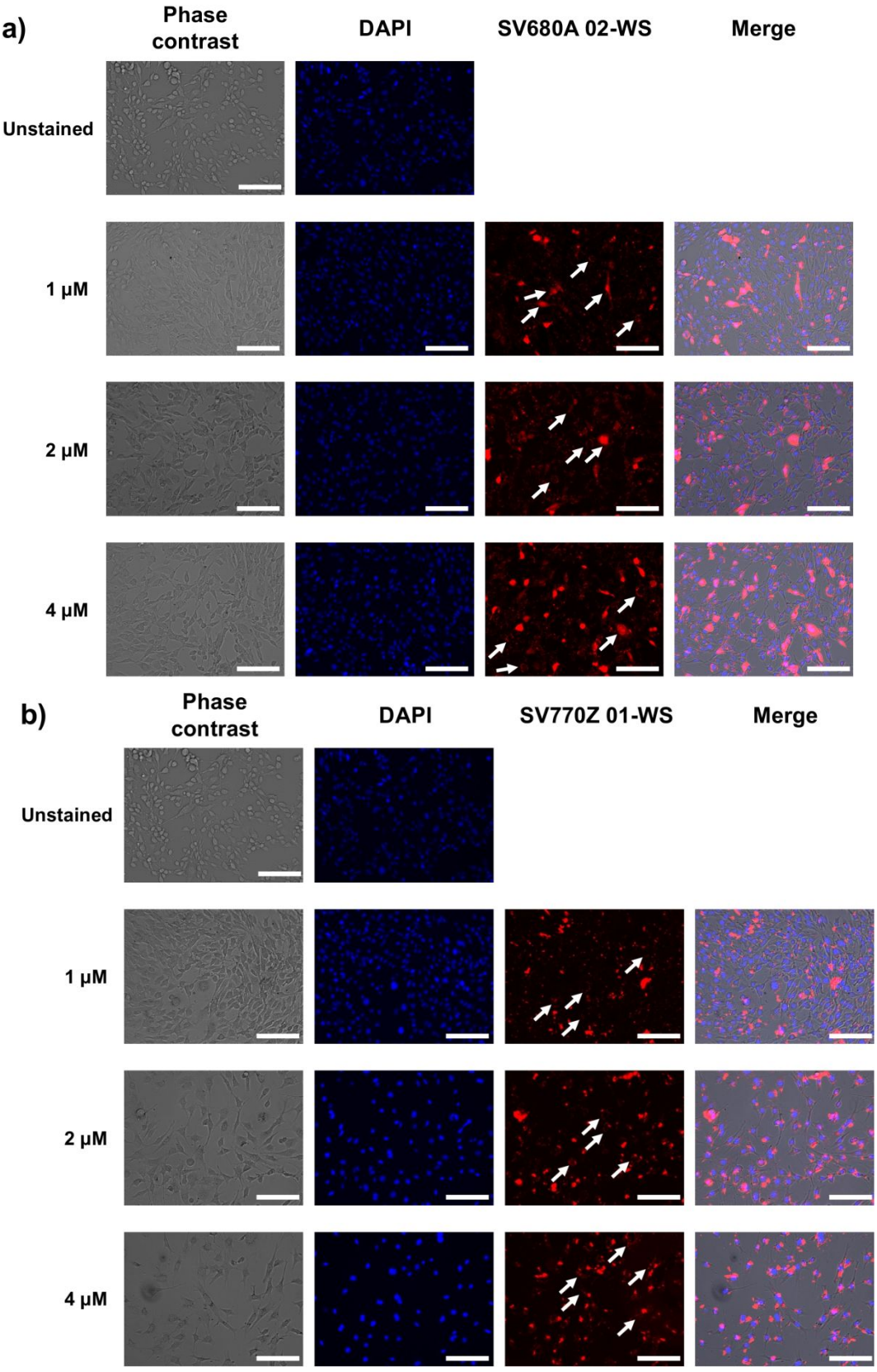
Cell culture, treatments, and assessment of cell apoptosis

The murine embryo fibroblast cell line 3T3-L1 (purchased from Lonza, Walkersville, MD) was cultured routinely in DMEM complete medium supplemented with 10% FBS, L-glutamine, and penicillin/streptomycin (all from Gibco, Grand Island, NY) under standard conditions at 37°C in a humidified atmosphere with 5% CO_2. The cells were seeded at a concentration of 100,000 cells per well in DMEM complete medium in a 6-well plate and incubated overnight. The dyes SV680A-02-WS Chitosan, SV645A-01-WS Chitosan, SV770Z-01-WS Chitosan, and SV620C-01-PEI were dissolved in DMEM complete medium, and their effects were comparatively evaluated in the 3T3-L1 cell line exposed to final concentrations of 4 μM, 2 μM, and 1 μM under standard conditions at 37°C in a humidified atmosphere with 5% CO_2. Untreated cells were used as a negative control. After 48 hours, adherent cells were recovered with 0.25% trypsin-EDTA and pooled with floating cells to analyse the degree of cell death and apoptosis in the entire cell population, using previously described methods [9] . To evaluate the cytotoxicity induced by the fluorescent chitosan and fluorescent PEI, the degree of cell apoptosis was quantified by Annexin V-FITC/PI staining (Beckman Coulter Inc., Brea, CA) using a FACSCalibur flow cytometer (BD Biosciences, San José, CA), as previously described [10]. Flow cytometric data analysis was performed using FlowJo software (Tree Star, Ashland, OR, USA). The results are reported as the percentage of total Annexin+ apoptotic cells relative to the total parental cell population.

Assessment of Chitosan Dye Uptake in 3T3-L1 Cells

The murine embryo fibroblast cell line, 3T3-L1, was seeded at a density of 30,000 cells per well in a 12-well plate containing complete DMEM medium and incubated overnight. SV680A-02-WS chitosan and SV770Z-01-WS chitosan were dissolved in complete DMEM medium at final concentrations of 4 μM, 2 μM, and 1 μM. After 48 hours incubations performed under standard conditions at 37°C with 5% CO_2, cells were washed with PBS and incubated with a 1:4000 dilution of HOECHST 33342 (10 mg/mL, 16.23 mM, Thermo Fisher Technologies) in DMEM complete medium for 20 minutes in a humidified incubator. Finally, the cells were washed three times with PBS, 500 μL of PBS was added, and images were acquired using an EVOS M5000 Cell Imaging System (Thermo Fisher

Technologies). All images were acquired with similar excitation intensity and detector sensitivity. Merged images were generated from the acquired images using Fiji software.



S37 Images of fluorescence channels and bright field channel of 3T3-L1 fibroblasts following 48 h incubation with SV680A-02-WS Chitosan (a) and SV770Z-01-WS Chitosan (b). Scale bar=100µm.

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