Supporting Information

Deep Red Blinking Fluorophore for Nanoscopic Imaging and Inhibition of *β*-Amyloid Peptide Fibrillation

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Methods

Chemicals and Materials. β-Amyloid (Aβ_{1-40}) (91.0%) was purchased from AnaSpec, Inc. (Fremont, CA, U.S.A.). NaH$_2$PO$_4$·2H$_2$O, Na$_2$HPO$_4$·12H$_2$O and ammonia solution (NH$_3$·H$_2$O) (25~28%) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Fluorescent beads (yellow-green fluorescent, 505/515 nm) were obtained from Invitrogen Ltd. (Eugene, Oregon, U.S.A.). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide (MTT), propidium iodide (PI) and Hoechst 33342 were obtained from Beyotime Biotechnology (Haimen, China). Fetal bovine serum (FBS) and Dulbecco's modified eagle medium (DMEM) were purchased from Invitrogen Corp (Carlsbad, CA, U.S.A.). Thioflavin T (ThT), Dimethyl sulfoxide (DMSO) and other chemicals not mentioned were purchased from Sigma-Aldrich (St. Louis, Mo, U.S.A.). Deionized (DI) water was used during the whole process of the experiments. All chemicals were obtained from commercial suppliers and used without further purification.

Synthesis of Carbazole-based Cyanine Fluorophores. The Knoevenagel reaction of 1-(2-(2-methoxyethoxy)ethyl)-4-methylquinolinium iodide (1) and 9-methyl-9H-carbazole-3-carbaldehyde (3) in the presence of piperidine in ethanol afforded carbazole-based cyanine, (*E*)-1-(2-(2-methoxyethoxy)ethyl)-4-(2-(9-methyl-9H-carbazol-3-yl)vinyl)quinolinium iodide (me-slg). The Knoevenagel reaction of 9-(2-(2-methoxyethoxy)-ethyl)-9H-carbazole-3-carbaldehyde (4) and the quinolinium halide 1 in the presence of piperidine in ethanol afforded carbazole-based cyanine, (*E*)-1-(2-(2-methoxyethoxy)ethyl)-4-(2-(9-(2-(2-methoxyethoxy)ethyl)-9H-carbazol-3

yl)vinyl)quinolinium iodide (slg). The Knoevenagel reaction of aldehyde 4 and the quinolinium halide 1 in the presence of piperidine in ethanol afforded carbazole-based cyanine, (E)-4-(2-(9-(2-(2-methoxyethoxy)ethyl)-9H-carbazol-3-yl)vinyl)-1-methylquinolinium iodide (slm). [1, 2]

The synthesis of 1. A solution mixture of lepidine (0.5 g, 3.5 mmol) and 1-iodo-2-(2-methoxyethoxy)ethane (3.22 g, 14 mmol) in acetonitrile (15 mL) was heated to reflux overnight. After cooling to room temperature, the solvent was removed. ^{1}H nuclear magnetic resonance (NMR) (400 MHz, DMSO-d$_6$) δ 9.28 (d, J = 6.0 Hz, 1H), 8.63 (d, J = 9.2 Hz, 1H), 8.55 (dd, J = 8.4 Hz, J = 1.2 Hz, 1H), 8.25 (td, J = 8.0 Hz, J = 1.2 Hz, 1H), 8.09-8.04 (m, 2H), 5.23 (t, J = 4.8 Hz, 2H), 3.95 (t, J = 5.2 Hz, 2H), 3.49 (m, 2H), 3.25 (m, 2H), 3.03 (s, 3H), 3.02 (s, 3H). ^{13}C NMR (400 MHz, DMSO-d$_6$) δ 158.9, 149.2, 136.9, 134.9, 129.5, 128.8, 127.1, 122.2, 119.5, 71.1, 69.5, 67.7, 58.0, 56.5, 19.8.

The synthesis of 3. n-BuLi (3.3 mL , 8.3 mmol) was added to a solution of 3-bromo-9-methyl-9H-carbazole (2) (1.8 g, 6.9 mmol) in dried THF (45 mL) at -78 °C. The resulting mixture was stirred at -78 °C for 1 h and then added with dried N,N-Dimethylformamide (DMF, 8 mL). The reaction mixture was allowed to warm to room temperature and stirred overnight before quenched with aqueous ammonia chloride solution. Water was added and extracted with ethyl acetate three times. The combined organic phase was washed with brine and dried over anhydrous sodium sulfate. After removing the solvent, the residue was purified by silica gel chromatography using ethyl acetate and petroleum ether (EA:PE = 1:4). ^{1}H NMR

(400 MHz, CDCl$_3$) δ 9.58 (s, 1H), 7.79 (s, 1H), 7.49 (d, J = 7.6 Hz, 1H), 7.41 (d, J = 8.8 Hz, 1H), 7.09 (t, J = 7.6 Hz,), 6.90 (t, J = 7.6 Hz, 1H), 6.77 (d, J = 8.0 Hz, 1H), 6.61 (d, J = 8.4 Hz, 1H), 3.00 (s, 3H). ^{13}C NMR (400 MHz, CDCl$_3$) δ 190.9, 143.2, 140.5, 127.4, 125.8, 122.7, 121.7, 119.6, 119.4, 108.3, 107.6, 28.0.

The synthesis of me-slg. A solution mixture of 1 (0.18 g, 0.5 mmol) and 3 (0.13 g, 0.6 mmol) and piperidine (0.1 mL) in ethanol (40 mL) was heated to reflux overnight. After cooling down to room temperature, the organic solvent was removed. The residue was purified by precipitation from methanol and ethyl acetate to afford me-slg (0.15 g) in 53% yield. ^{1}H NMR (400 MHz, DMSO-d$_6$) δ 9.19 (d, J = 6.8 Hz, 1H), 9.15 (d, J = 8.8 Hz, 1H), 8.89 (s, 1H), 8.56 (d, J = 8.8 Hz, 1H), 8.52 (d, J = 6.8 Hz, 1H), 8.47 (d, J = 16 Hz, 1H), 8.39 (d, J = 16 Hz, 1H), 8.27-8.23 (m, 5H), 8.16 (dd, J = 8.8 Hz, J = 1.6 Hz, 1H), 8.06 (t, J = 7.6 Hz, 1H), 7.77 (d, J = 8.8 Hz, 1H), 7.68 (d, J = 8.4 Hz, 1H), 7.56 (t, J = 7.6 Hz, 1H), 7.33 (t, J = 7.6 Hz, 1H), 5.16 (t, J = 4.8 Hz, 2H), 3.97 (t, J = 4.8 Hz, 2H), 3.96 (s, 3H), 3.53 (m, 2H), 3.29 (m, 2H), 3.06 (s, 3H). ^{13}C NMR (400 MHz, DMSO-d$_6$) δ 153.5, 147.8, 145.4, 142.4, 141.3, 138.1, 134.9, 128.9, 127.6, 126.8, 126.7, 126.6, 126.5, 122.7, 122.1, 121.9, 120.5, 119.9, 119.3, 116.2, 114.8, 109.9, 71.2, 69.6, 67.8, 58.1, 55.9, 29.4. HRMS (matrix-assisted desorption ionization-time-of-flight (MALDI-TOF)) m/z Calcd for C$_{29}$H$_{29}$N$_2$O$_2$ 437.2223, Found 437.2214 [M]$^+$.

The synthesis of slg. A solution mixture of 1 (0.30 g, 0.8 mmol) and 4 (0.33 g, 1.1 mmol) and piperidine (0.1 mL) in ethanol (40 mL) was heated to reflux overnight. After cooling down to room temperature, the organic solvent was removed. The

residue was purified by precipitation from methanol and ethyl acetate to afford slg (0.25 g) in 48% yield. ^{1}H NMR (400 MHz, DMSO-d6) δ 9.18 (d, J = 6.8 Hz, 1H), 9.14 (d, J = 8.0 Hz, 1H), 8.87 (s, 1H), 8.55 (d, J = 8.8 Hz, 1H), 8.51 (d, J = 6.8 Hz, 1H), 8.45 (d, J = 16 Hz, 1H), 8.36 (d, J = 16 Hz, 1H), 8.24 (d, J = 7.2 Hz, 2H), 8.13 (d, J = 7.6 Hz, 1H), 8.05 (t, J = 7.6 Hz, 1H), 7.77 (d, J = 8.4 Hz, 1H), 7.69 (d, J = 8.0 Hz, 1H), 7.52 (t, J = 7.2 Hz, 1H), 7.31 (t, J = 7.2 Hz, 1H), 5.15 (t, J = 5.2 Hz, 2H), 4.63 (t, J = 4.8 Hz, 2H), 3.97 (t, J = 5.2 Hz, 2H), 3.84 (t, J = 4.8 Hz, 2H), 3.53 (t, J = 4.8 Hz, 2H), 3.48 (t, J = 4.8 Hz, 2H), 3.32-3.29 (m, 4H), 3.11 (s, 3H), 3.06 (s, 3H). ^{13}C NMR (400 MHz, DMSO-d6) δ 153.5, 147.7, 145.3, 142.2, 140.9, 138.1, 134.8, 128.8, 127.4, 126.7, 126.4, 122.8, 122.2, 121.8, 120.4, 119.8, 119.2, 116.2, 114.8, 110.4, 110.3, 71.2, 71.1, 69.8, 69.6, 68.8, 67.8, 58.1, 58.0, 55.9, 42.9. HRMS (MALDI-TOF) m/z Calcd for $C_{33}H_{37}N_2O_4$ 525.2747, Found 525.2747 $[M]^+$.

The synthesis of slm. A solution mixture of 1 (0.23 g, 0.8 mmol) and 4 (0.33 g, 1.1 mmol) and piperidine (0.1 mL) in ethanol (40 mL) was heated to reflux overnight. After cooling down to room temperature, the organic solvent was removed. The residue was purified by precipitation from methanol and ethyl acetate to afford slm (0.24 g) in 47% yield. ^{1}H NMR (400 MHz, DMSO-d6) δ 9.27 – 9.22 (m, 1H), 9.12 (d, J = 8.5 Hz, 1H), 8.84 (s, 1H), 8.48 (dd, J = 6.4, 3.0 Hz, 1H), 8.44 – 8.34 (m, 3H), 8.25 (dd, J = 13.9, 7.2 Hz, 2H), 8.15 – 8.01 (m, 3H), 7.76 (dd, J = 8.6, 2.3 Hz, 1H), 7.69 (d, J = 8.2 Hz, 1H), 7.55 – 7.50 (m, 1H), 7.31 (t, J = 7.4 Hz, 1H), 4.61 (d, J = 4.7 Hz, 2H), 4.50 (d, J = 2.5 Hz, 3H), 3.84 (t, J = 5.1 Hz, 2H), 3.49 – 3.47 (m, 2H), 3.31 (dd, J = 5.5, 3.9 Hz, 2H), 3.13 – 3.10 (m, 3H). ^{13}C NMR (101 MHz, DMSO-d6) δ 153.47,

147.94, 145.30, 142.57, 141.36, 139.25, 135.33, 129.42, 127.79, 127.20, 126.90, 126.88, 126.55, 123.25, 122.65, 122.20, 120.86, 120.32, 119.70, 116.63, 115.54, 110.89, 110.78, 71.73, 70.26, 69.32, 58.54, 44.88, 43.37. HRMS (ESI-TOF) m/z calcd for $C_{29}H_{29}N_2O_2$ 437.2224, Found 437.2223 $[M]^+$.

Characterizations of Me-Slg, Slg and Slm. Ultraviolet-visible (UV-vis) absorption spectra of me-slg, slg and slm (10 μM) were collected using a UV-2450 UV-vis spectrophotometer (Shimadzu, Tokyo, Japan). The spectra were collected using a quartz cuvette having 2 mm path length and 0.7 mL volume. Fluorescence spectra of me-slg, slg and slm (10 μM) were recorded on the F-4600 fluorescence spectrometer (Hitachi, Tokyo, Japan). All measurements were repeated at least three times.

^{1}H NMR spectra were recorded at 400 MHz and ^{13}C NMR spectra were recorded at 100 MHz on a Bruker advanced-III 400 NMR spectrometer (Bruker Corporation, Karlsruhe, Germany). High resolution mass spectrometry (HRMS) was recorded on an Ultraflex III MALDI-TOF/TOF mass Spectrometer (Bruker Daltonics, Bremen, Germany).

Preparation of β-Amyloid Aggregates. Monomeric Aβ_{1-40} peptides stock solution was prepared by dissolving 1 mg of Aβ_{1-40} powder in 400 μL of ice-cold $NH_3 \cdot H_2O$ (0.02%) without any purification and stored at -20 °C before use.[3] For Aβ_{1-40} fibrillation experiments, 5μL stock solution of monomeric Aβ_{1-40} peptides (500 μM) was diluted to 45 μL of phosphate buffer (PB, 25 mM, pH = 7.4) solution, and then incubated at 37 °C for different time. Prior to use, Aβ_{1-40} aggregates (50 μM) were

stored at 4 °C.

PI and Hoechst 33342 Co-Staining Assay. Rat pheochromocytoma (PC12) cells were used as neuronal cell model.[4] $A\beta_{1-40}$ monomers (50 μM) were firstly incubated in PB solution at 37 °C for 0, 10 and 60 min, respectively. PC12 cells were seeded at the confocal cell culture dish in DMEM containing 10% FBS and incubated at 37 °C for 24 h. After the cells were co-incubated with above $A\beta_{1-40}$ aggregates (with a final concentration of 10 μM) for another 24 h, the cell culture medium was replaced with fresh medium containing PI (200 μL, 15 nM). After 30 min, the culture medium was removed and washed three times with phosphate buffered saline (PBS) solution (10 mM, pH = 7.4). To stain the nucleus of the cell, the cell was further incubated with the cell culture medium with Hoechst 33342 (200 μL) for 30 min at 37 °C. After being washed three times with PBS solution, the cells were photographed by confocal laser scanning microscopy (CLSM, Nikon A1, Nikon Co. Ltd., Tokyo, Japan).

Cytotoxicity Assay. Cytotoxicity assay was characterized by the MTT assay.[5, 6] PC12 cells were incubated in the 96-well plates with a density of about 3×10^4 cells per well and incubated for 24 h at 37 °C. After being washed with PBS solution three times, the cells were added with fresh medium containing different samples (*e.g.*, different concentrations of me-slg, slg and slm (0.01, 1, 10, and 50 μM), $A\beta_{1-40}$ aggregates at different growth stages (with a final concentration of 10 μM), $A\beta_{1-40}$ fibrils (with a final concentration of 10 μM) and the mixture of me-slg (with a final concentration of 2 μM) and $A\beta_{1-40}$ (with a final concentration of 10 μM). The culture medium was removed after co-incubated for 24 h, and then 20 μL of MTT solution (5

mg mL^{-1}) was injected to each well and cultured for another 4 h. Finally, the supernatant was discarded and the crystal on the bottom was dissolved with 150 μL of DMSO. The optical density (OD) value at 490 nm was measured by a Tecan Sunrise microplate reader (Tecan Austria GmbH, Grödlg, Austria) to calculate the cell viability. The relative cell viability was defined as: Cell viability (%) = $OD_{sample}/OD_{control}$, where OD_{sample} and $OD_{control}$ were obtained in the presence/absence of samples, respectively. The data were presented as mean ± standard deviation (SD), based on five measurements and examined for their statistical significance of difference with Student's t-test.

Supporting Figures

R = CH₃OCH₂CH₂OCH₂CH₂ (me-slg)
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    CH₃ (slm)
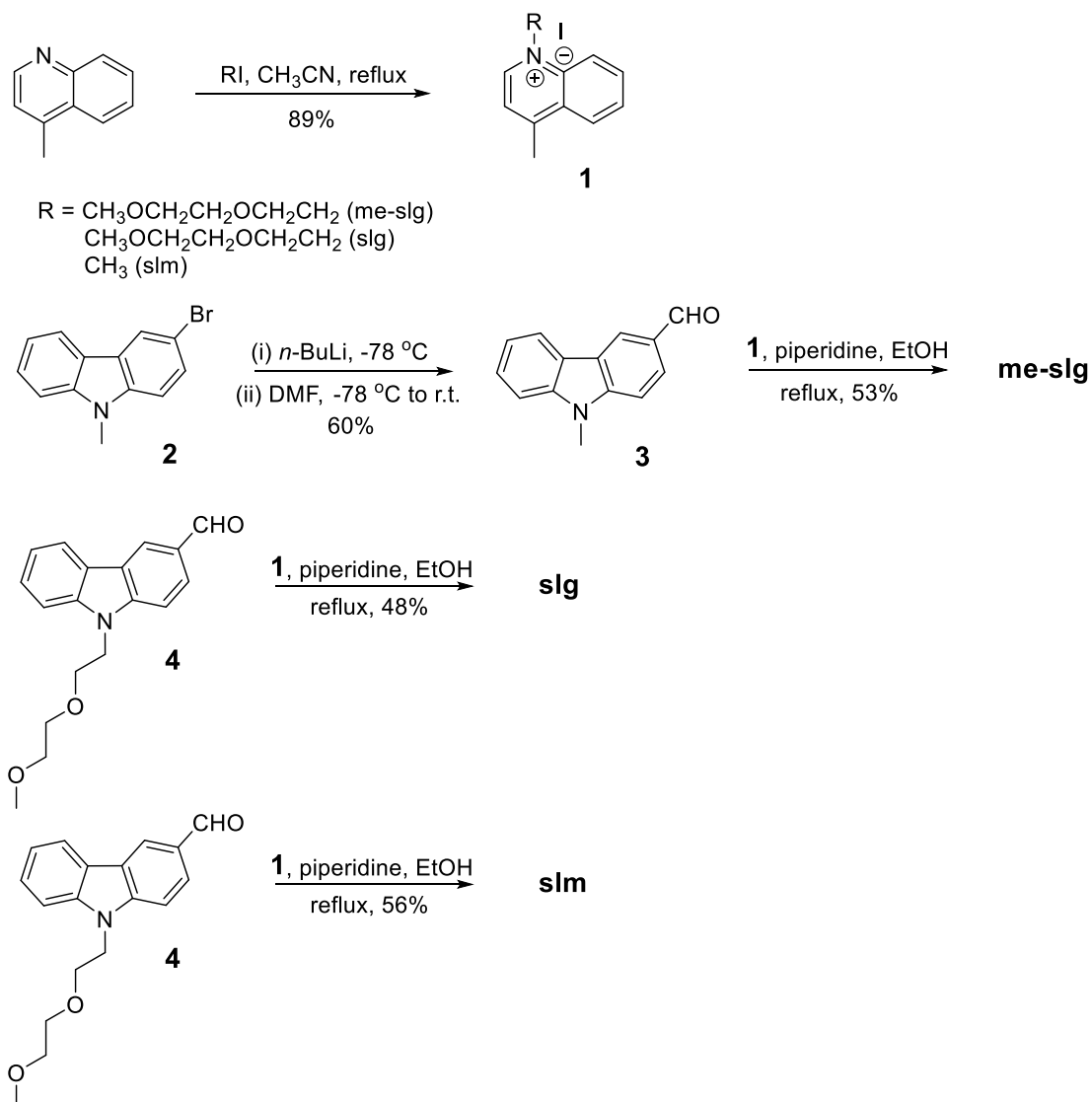
Figure S1. The synthesis of me-slg, slg and slm.
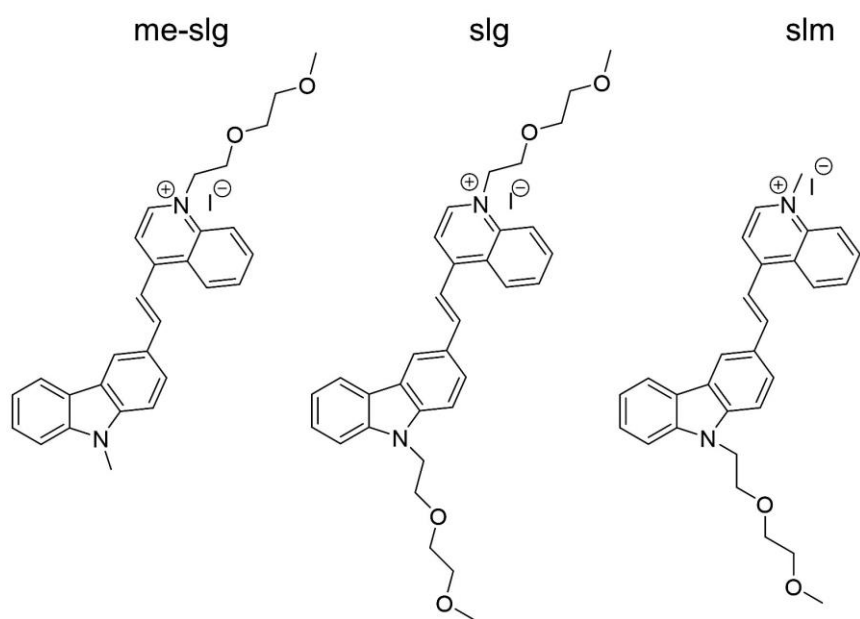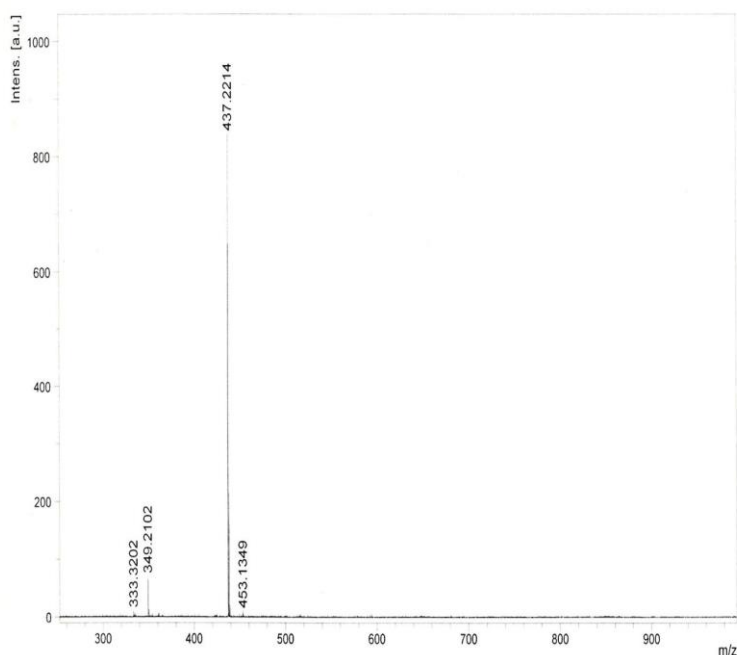
Figure S2. The molecular structure of me-slg, slg and slm.
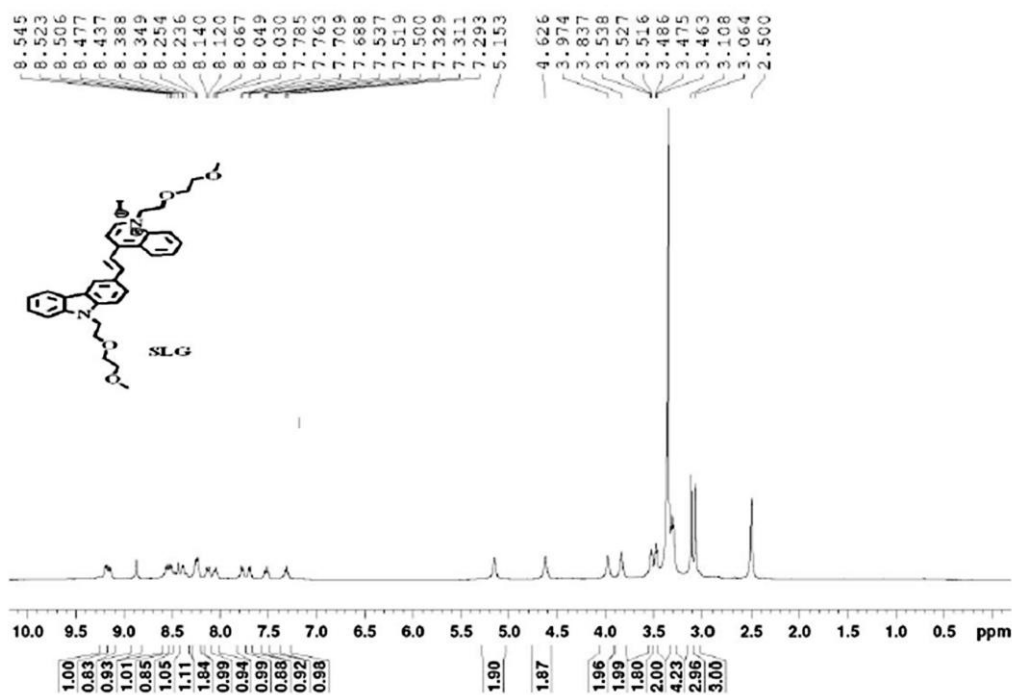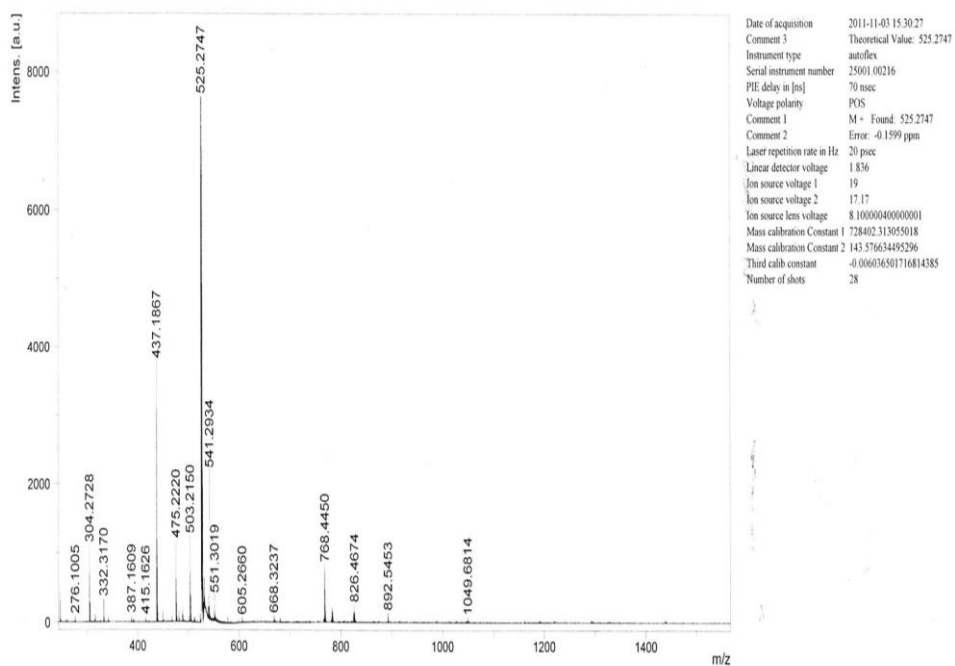
Me-slg spectra
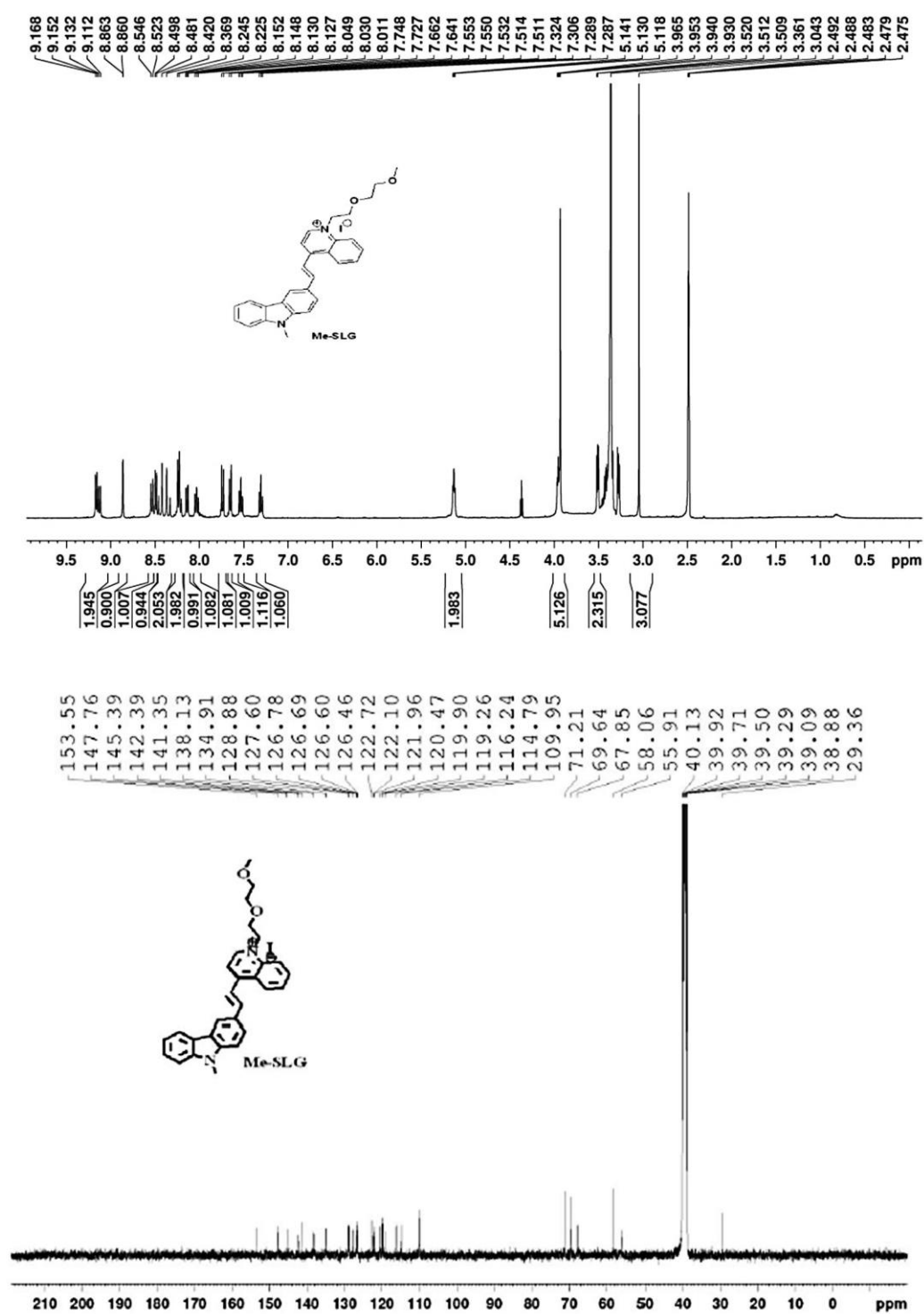
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Slg spectra

NMR chemical shifts (ppm): 153.48, 147.66, 145.29, 142.17, 140.90, 140.07, 134.83, 128.80, 127.39, 126.73, 126.38, 122.80, 122.19, 121.83, 120.38, 119.84, 119.18, 116.19, 114.75, 110.41, 110.30, 71.24, 71.15, 69.78, 69.62, 68.83, 67.82, 58.04, 58.01, 55.86, 42.91, 40.13, 39.92, 39.71, 39.50, 39.30, 39.09, 38.88

SLG

210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm

Intens. [a.u.]

Mass spectrum peaks (m/z): 276.1005, 304.2728, 332.3170, 387.1609, 415.1626, 437.1867, 475.2220, 503.2150, 525.2747, 541.2934, 551.3019, 605.2660, 668.3237, 768.4450, 826.4674, 892.5453, 1049.6814

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Slm spectra
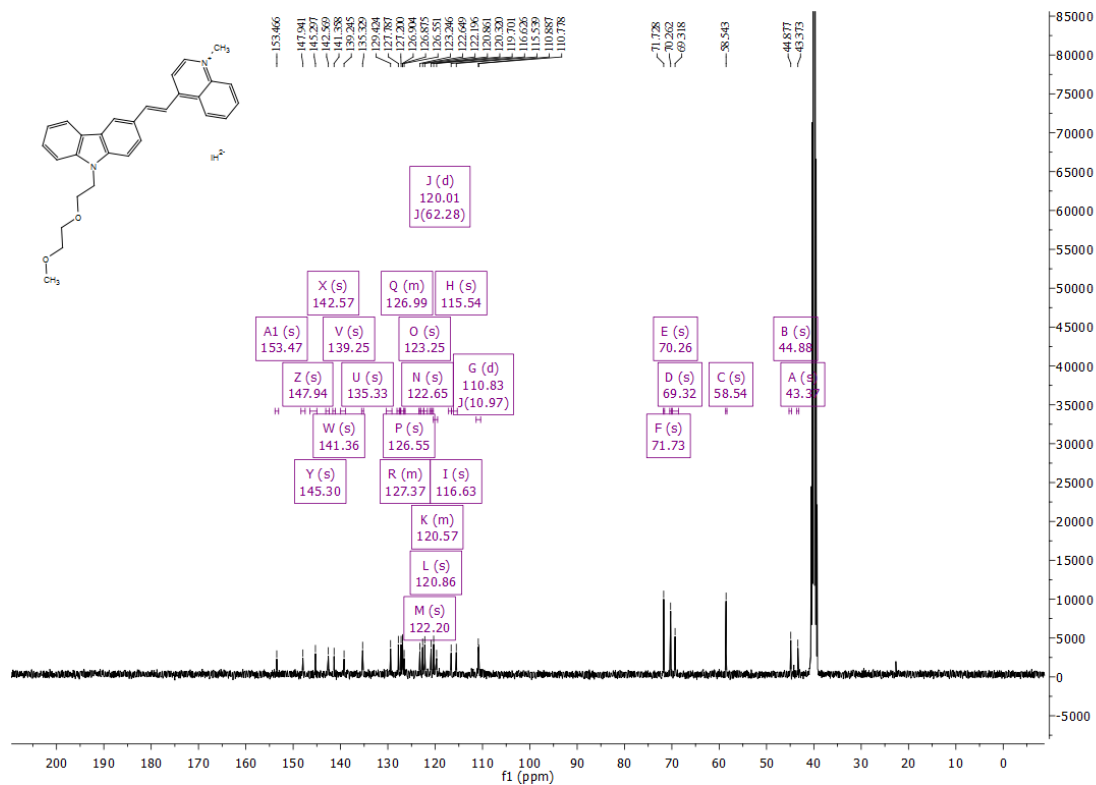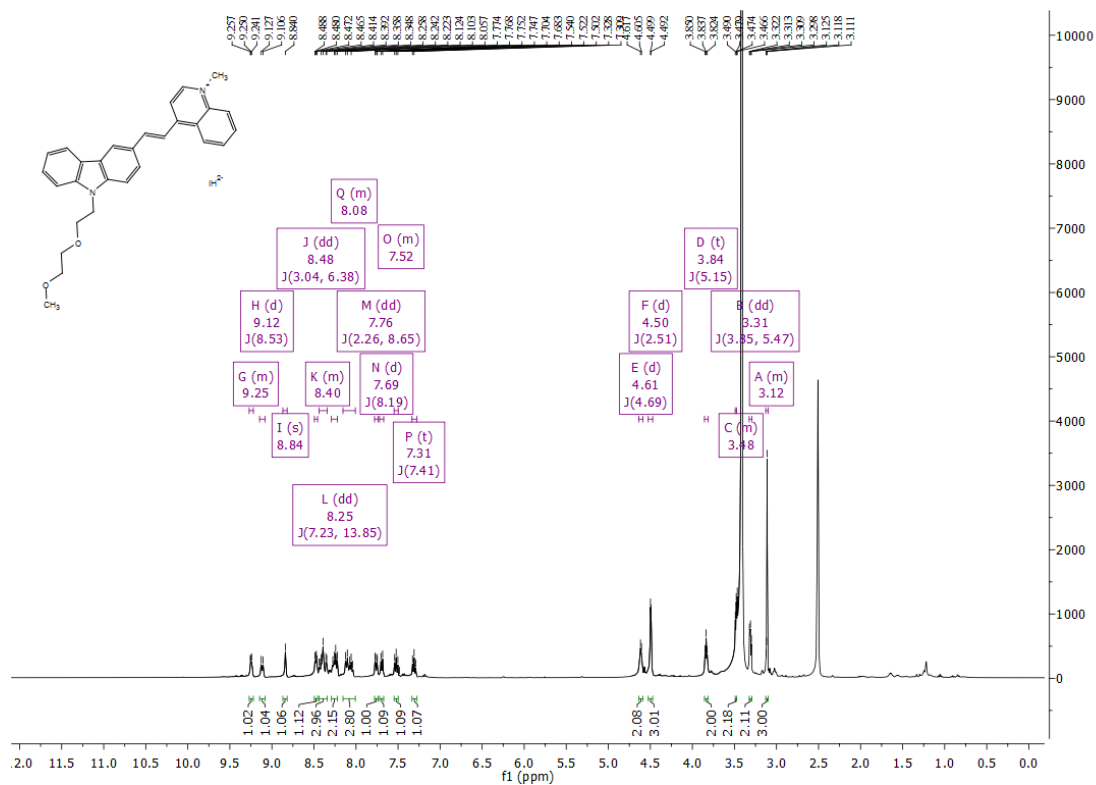
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C	4	10	30		S	2	0	0							
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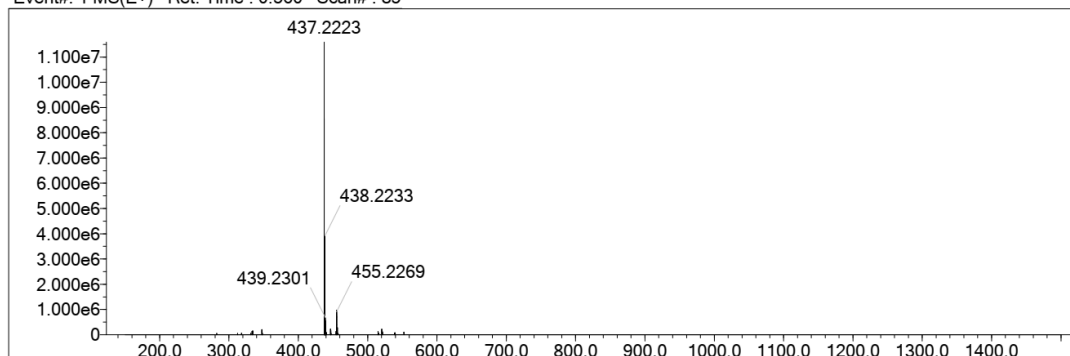
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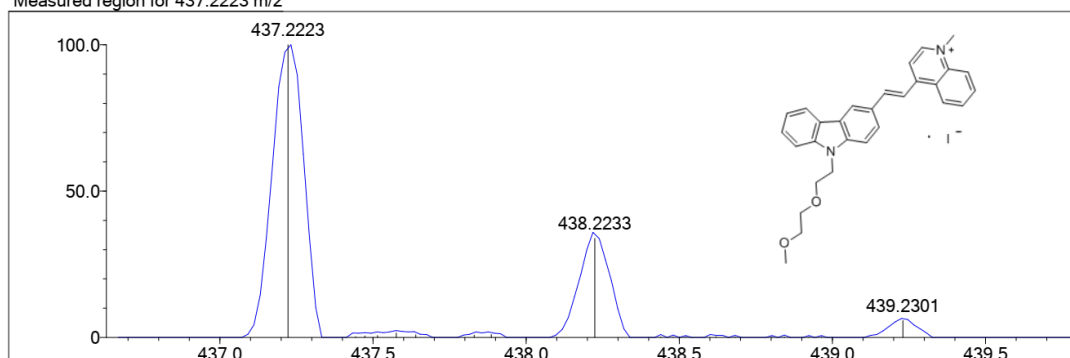
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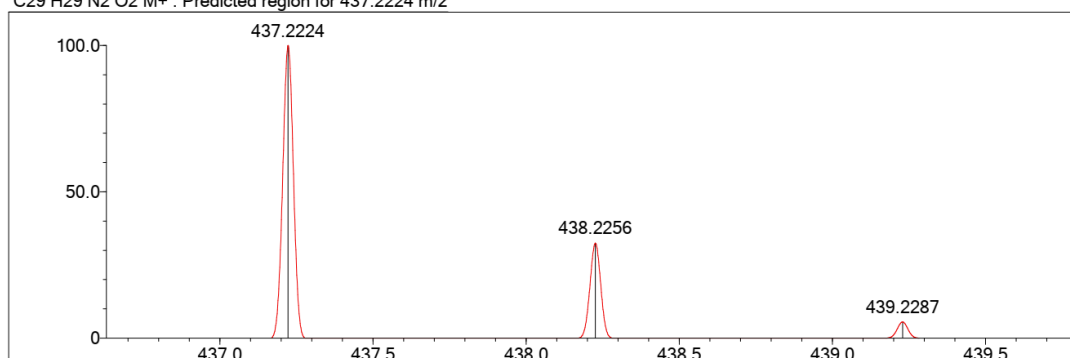
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Figure S3. ^{1}H NMR, ^{13}C NMR and HRMS of me-slg, slg and slm.
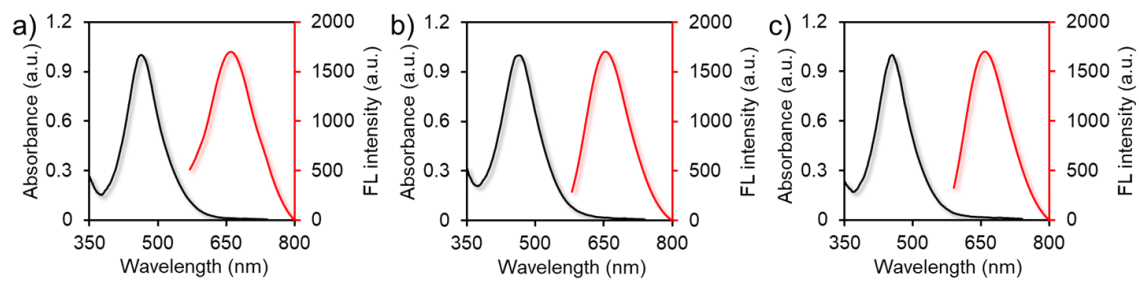
Figure S4. The absorption (black line) and fluorescence emission (red line) spectra of (a) me-slg, (b) slg and (c) slm.
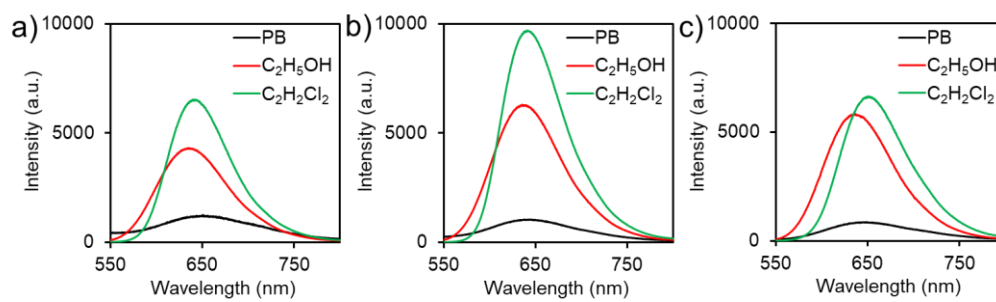
Figure S5. Fluorescence spectra of (a) me-slg, (b) slg and (c) slm in PB, ethanol and methylene chloride (10 μM).
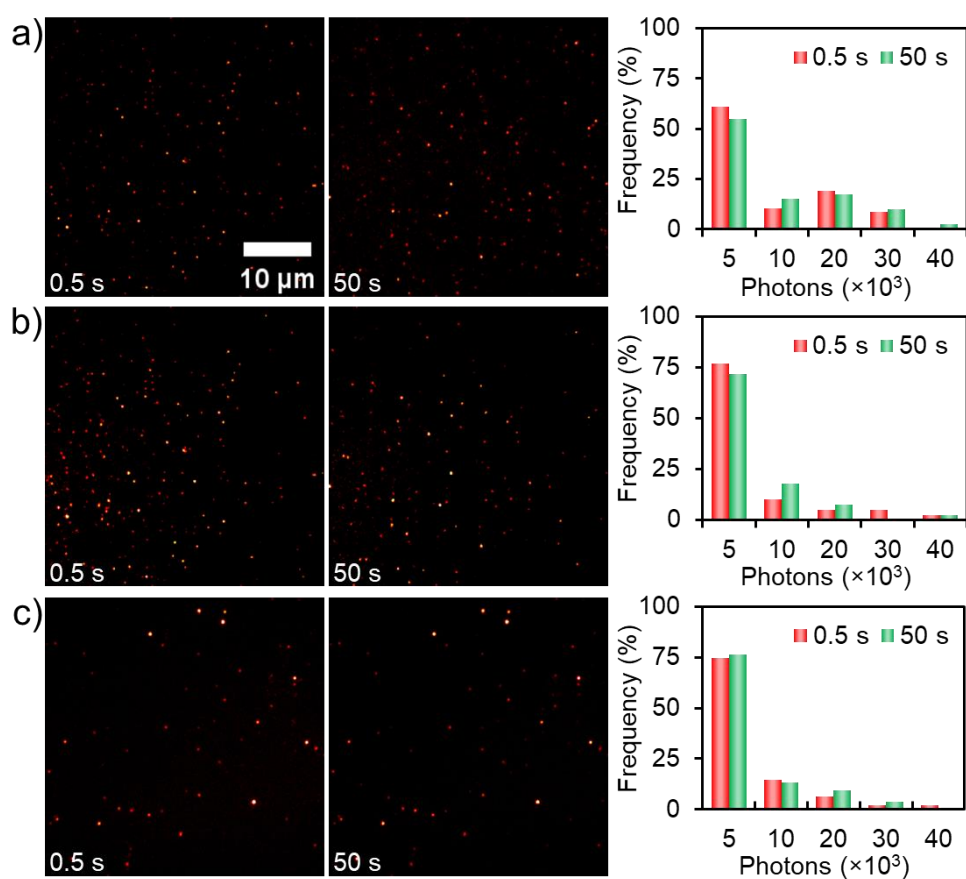
Figure S6. Representative fluorescence images of (a) me-slg, (b) slg and (c) slm at 0.5 and 50 s. The statistically analyzed photon counts from individual me-slg, slg and slm, respectively.
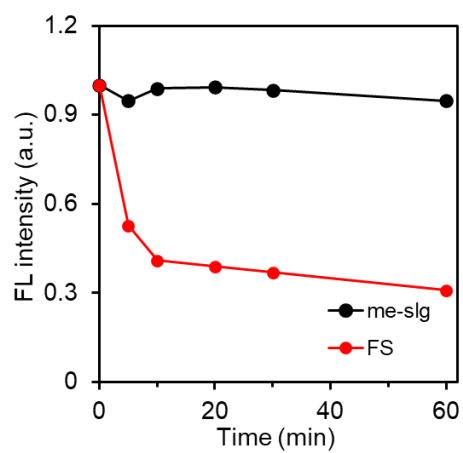
Figure S7. Fluorescence intensity plots of me-slg and fluorescein sodium (FS) in water under continuous illumination (22 W cm^{-2}).
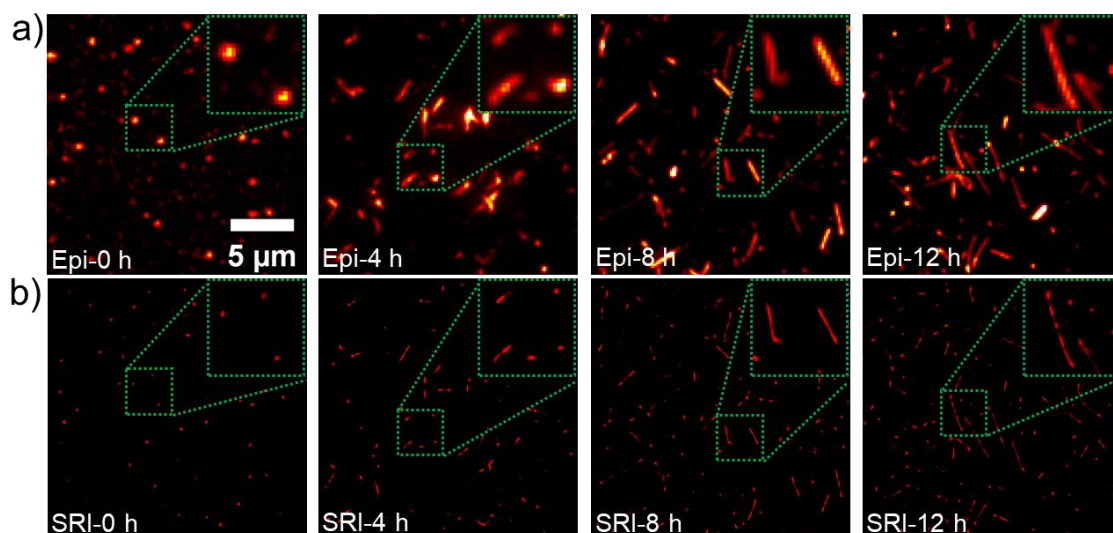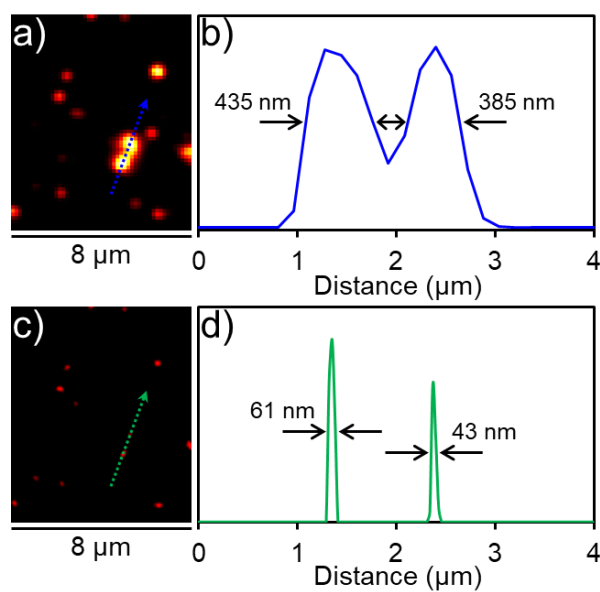
Figure S8. (a) and (b) Representative conventional fluorescence images and the corresponding reconstructed nanoscopic fluorescence images of Aβ_{1-40} aggregates stained by me-slg at 0, 4, 8 and 12 h during fibrillation process.
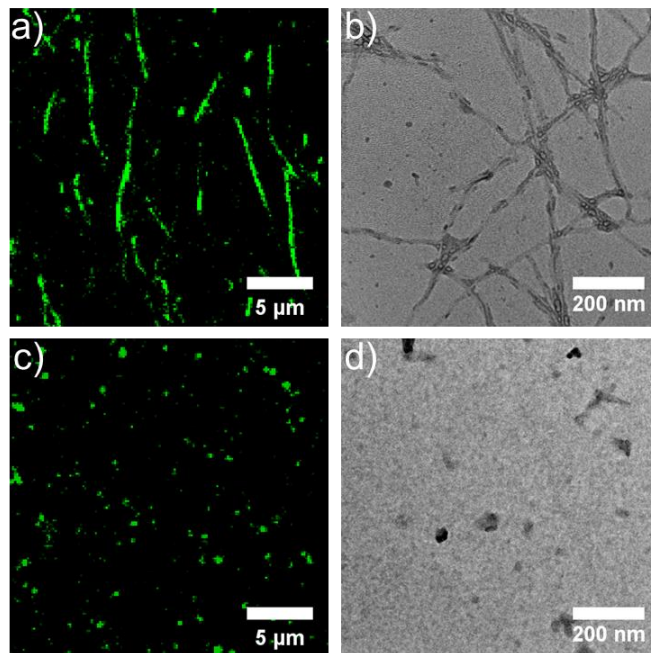
Figure S9. (a) and (b) The fluorescence and TEM images of $A\beta_{1\text{-}40}$ fibrils growing in PB solution at 37 °C for 12 h. (c) and (d) The fluorescence and TEM images of monomeric $A\beta_{1\text{-}40}$ peptides treated with me-slg in PB solution at 37 °C for 12 h.
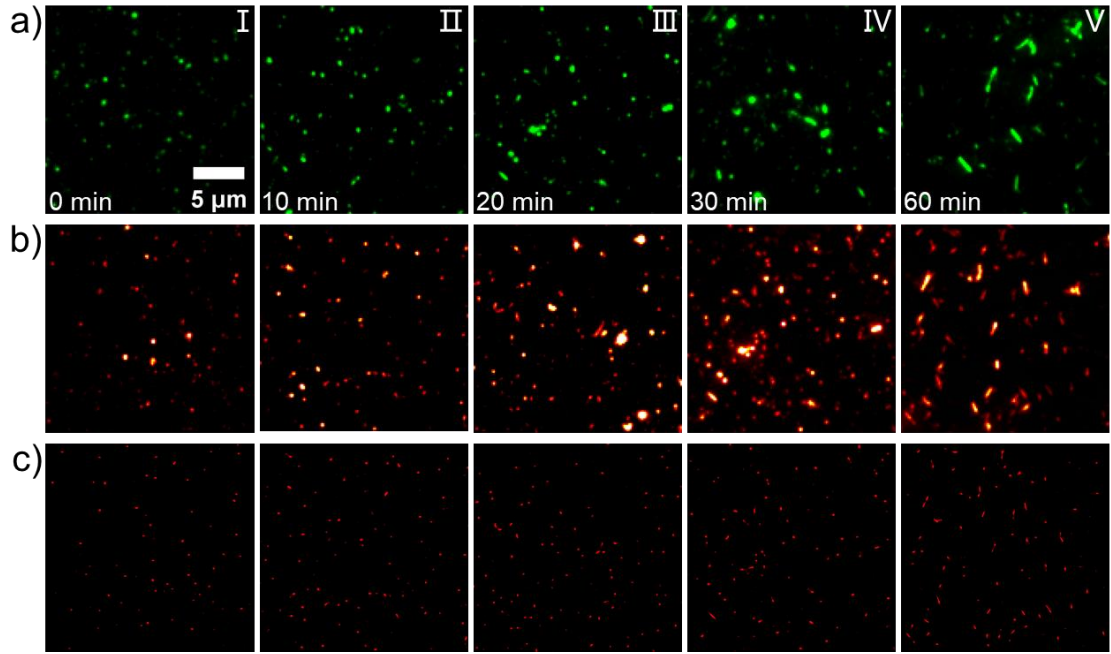
Figure S10. (a) Representative epi-fluorescence images of early stage $A\beta_{1-40}$ aggregates at different growth processes stained by me-slg in DMEM. (b) and (c) Representative epi-fluorescence images and the corresponding reconstructed nanoscopic fluorescence images of early stage $A\beta_{1-40}$ aggregates at different growth processes after growing for another 24 h and stained by me-slg in DMEM.

Figure S11. (a) and (c) Representative conventional fluorescence image and the corresponding reconstructed nanoscopic fluorescence image of the Aβ_{1-40} peptides treated with me-slg (I:P = 0.2) for 12 h at 37 ℃. (b) and (d) The corresponding intensity profiles of the blue and green lines in (a) and (c), respectively.
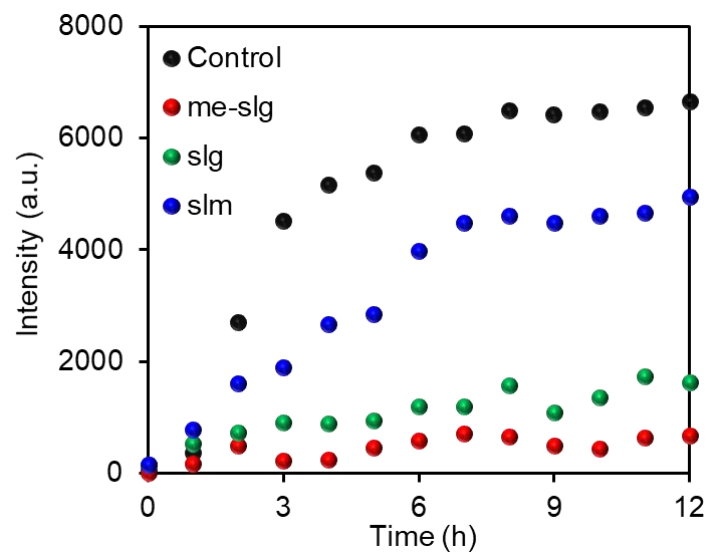
Figure S12. The time dependent ThT fluorescence intensity measurements of A$\beta_{1\text{-}40}$ (with monomer concentration of 50 μM) without (black) and with me-slg, slg and slm (red, green and blue).
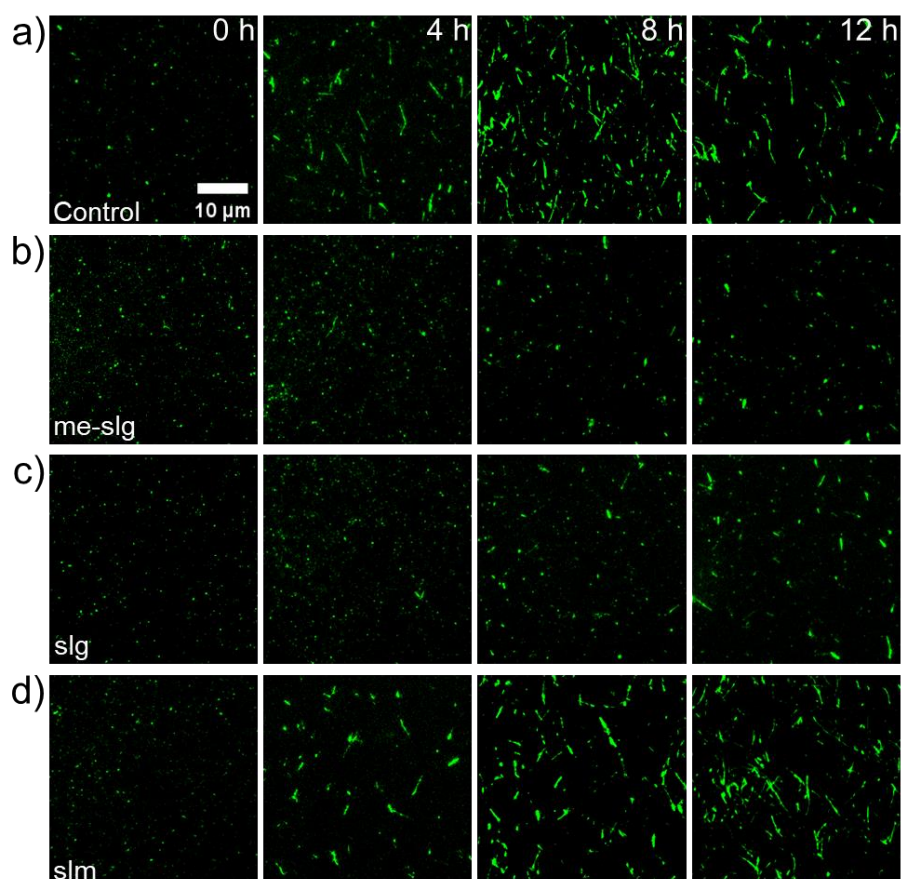
Figure S13. (a) The fluorescence microscopic characterizations of Aβ_{1-40} fibrillation process at 0, 4, 8 and 12 h. (b)-(d) The fluorescence microscopic characterizations of the inhibition effect of me-slg, slg and slm (I:P = 0.2) on Aβ_{1-40} fibrillation at 0, 4, 8 and 12 h.
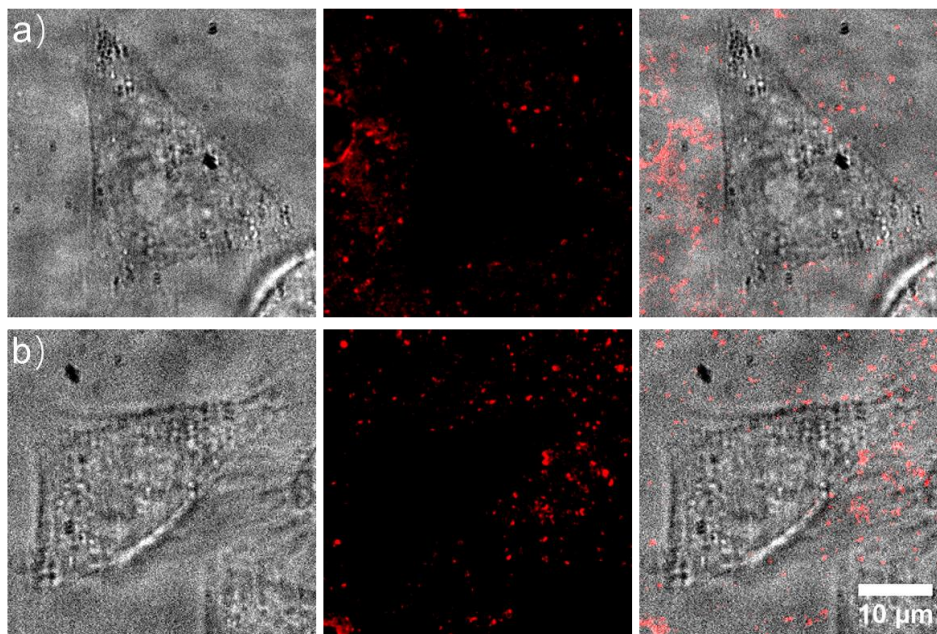
Figure S14. (a) and (b) Fluorescence microscopic characterizations of the inhibition effect of me-slg (I:P = 0.2) on Aβ_{1-40} fibrillation before and after incubating 12 h with PC12 cells. From left to right are the bright-field, fluorescence and the merged microscopic images.
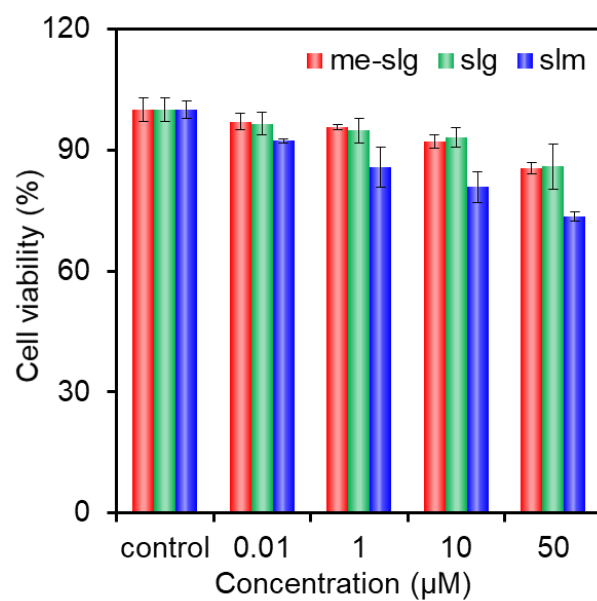
Figure S15. The cell viability of PC12 cells after being treated with different concentrations of me-slg, slg and slm (0.01, 1, 10, and 50 μM) for 24 h.
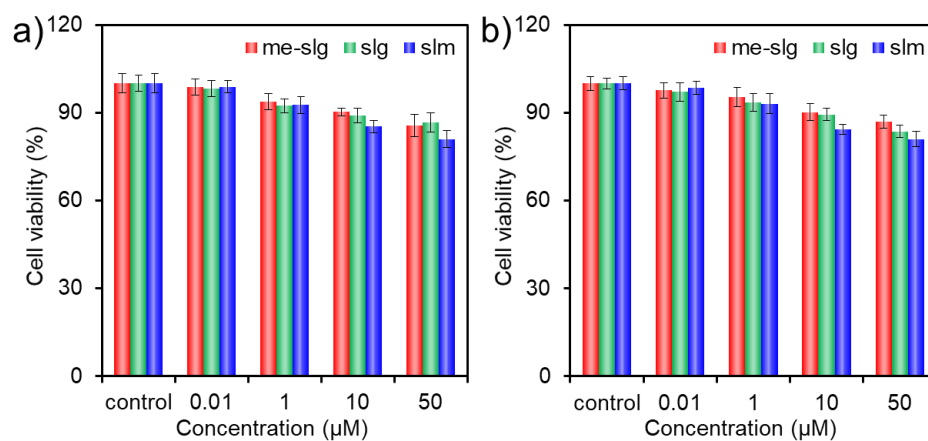
Figure S16. The cell viability of Hela and MCF-7 cells after being treated with different concentrations of me-slg, slg and slm (0.01, 1, 10, and 50 μM) for 24 h.
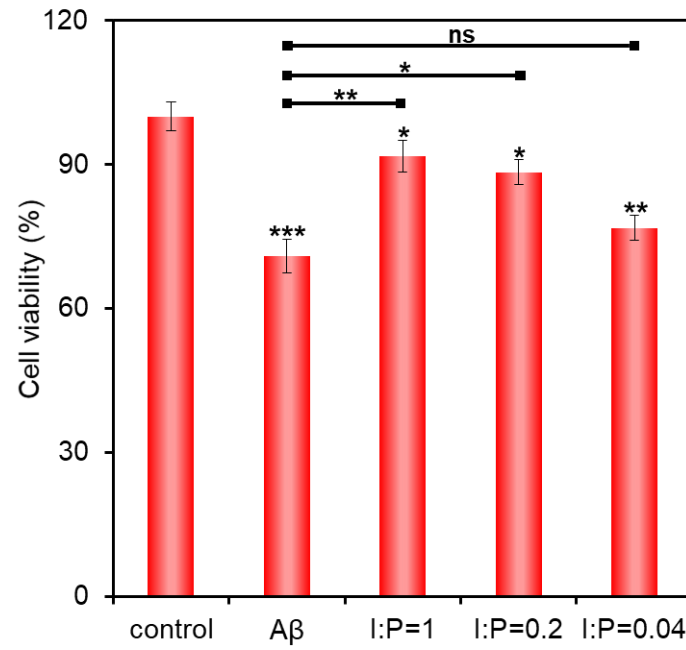
Figure S17. Neuroprotective effect of different dosage of me-slg against the toxicity induced by $A\beta_{1-40}$ aggregates (I:P = 1, 0.2 and 0.04) on PC12 cells (Student's t-test, n = 5, p*<0.05, p**<0.01, p***<0.001, ns: no significant).
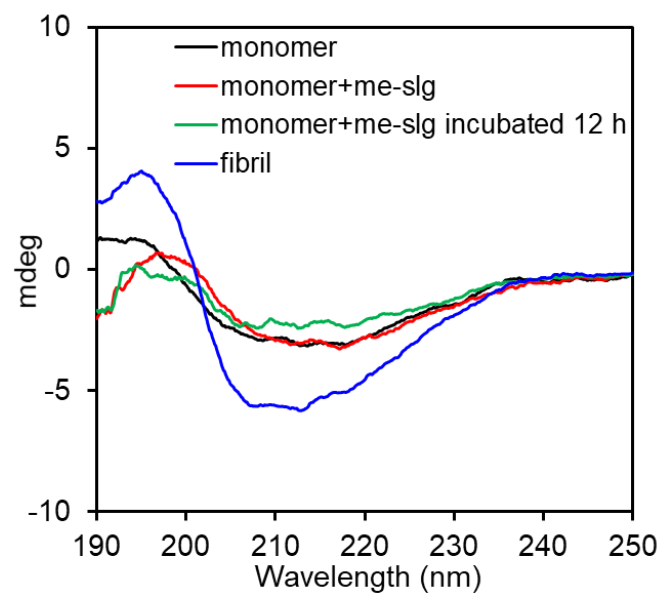
Figure S18. CD spectra of monomeric Aβ_{1-40} peptides (50 μM, black curve), monomeric Aβ_{1-40} peptides with me-slg at 0 h (I:P = 0.2, red curve), monomeric Aβ_{1-40} peptides with me-slg after co-incubated for 12 h (green curve), and Aβ_{1-40} fibril (blue curve).

Table S1. Optical properties of me-slg, slg and slm in PB solution.

	λ_{max}^{abs} / nm ($\varepsilon_{max} \times 10^4 / M^{-1}cm^{-1}$)	λ_{max}^{em} [a] (Stokes shift) / nm
me-slg	465 (0.90)	682 (217)
slg	467 (1.90)	674 (207)
slm	455 (1.59)	676 (221)

[a] excited at the maximum of the absorption.

Table S2. Fluorescence enhancement and Stokes shift of me-slg, slg and slm after binding with Aβ_{1-40} monomers and fibrils.

	me-slg	slg	slm
F$_{monomer}$/F$_{fluorophore}$ [a]	2.31	1.58	1.57
F$_{fibril}$/F$_{fluorophore}$ [b]	6.64	1.97	2.07
Stokes shift $_{fluorophore + monomer}$ (nm)	17	19	24
Stokes shift $_{fluorophore + fibril}$ (nm)	24	24	28

[a] Fluorescence enhancement ratio measured at 10 equivalents of Aβ_{1-40} monomers content.

[b] Fluorescence enhancement ratio measured at 10 equivalents of Aβ_{1-40} fibrils content.

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