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Electronic Supplementary Material (ESI).

Supporting Information

Bioreducible cross-linked core polymer micelles enhance *in vitro* activity of methotrexate in breast cancer cells

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Table of Contents

1. Measurements....3
   1.1. Nuclear magnetic resonance (NMR) spectroscopy....3
   1.2. Fourier transform infrared (FTIR) spectroscopy....3
   1.3. Size exclusion chromatography (SEC)....3
   1.4. Ultraviolet-visible spectroscopy (UV-Vis)....4
   1.5. Fluorescence spectroscopy....4
   1.6. Dynamic Light Scattering (DLS)....4
   1.7. Zeta potential measurement....4
   1.8. Transmission electron microscopy (TEM)....4
   1.9. Electrospray ionization mass spectrometry....5
   1.10. Confocal laser scanning microscopy....5
2. Supplementary Methods....5
   2.1. Synthesis of Oregon-green....5
   2.2. Critical micelle concentration (CMC)....5
   2.3. Cell culture....6
3. Supplementary Figures....6

List of Supplementary Figures

Fig. S1. ^{13}C NMR spectrum of mPEG-b-poly(εCL-co-αClεCL) in $CDCl_3$.....6

Fig. S2. ^{13}C NMR spectrum of mPEG-b-poly(εCL-co-αN_3CL) in $CDCl_3$.....7

Fig. S3. SEC traces of mPEG, mPEG-b-poly(εCL-co-αClεCL), and mPEG-b-poly(εCL-co-αN_3CL) in $CHCl_3$. 7

Fig.S4 (A) Schematic representation for the synthesis of mPEG-PCL, (B) 1H NMR spectrum, and (C) SEC traces in CHCl3 (PS calibration). 8

Fig. S5. ^{13}C NMR spectrum of redox-responsive bis-alkyne-ethyl disulfide crosslinker. 8

Fig. S6 Size distribution of mPEG-PCL micelles measured by DLS. 9

Fig. S7. CMC plot for mPEG-b-poly(εCL-co-αN_3εCL). 9

Fig. S8. Zeta potential measurements of empty and MTX-loaded micelles. (A) Empty micelles, (B) MTX-loaded micelles, and (C) MTX-loaded crosslinked micelles. 10

Fig. S9. ^{1}H NMR spectrum of Oregon-green. 11

Fig. S10. Electrospray ionization mass spectrum of Oregon-green. 11

Fig. S11. Size distribution of dye-loaded micelles measured by DLS. (A) Oregon-green loaded un-crosslinked micelles, and (B) Oregon-green loaded core-crosslinked micelles.....12

Fig, S12. Fluorescence microscopy analysis of apoptotic and dead cells in MCF7 breast cancer cells cultured for 48 hours with (A) Medium control and (B) Medium-DMSO control. Hoechst 33342 dye was used to detect apoptotic cells, whereas propidium iodide (PI) was used to detect dead cells. Scale bars ~ 200 µm.....13

Fig. S13. Fluorescence microscopy analysis of apoptotic and dead cells in MCF7 breast cancer cells cultured with (A) Free MTX as a control. a, b, c, d, and e, represents the various concentrations of MTX ranging from 4.6 µg/mL, 11.5 µg/mL, 23 µg/mL, 36 µg/mL, and 50 µg/mL, respectively. (B) The percentage of live, apoptotic, and dead cells as calculated by Infinity Analyze software (Infinity Analyze 3[R], Lumenera Corporation). Error bars represent standard deviation. n=3. Scale bars ~ 200 µm. 14

Fig. S14. Flow cytometry analysis to measure the apoptotic and dead cells in MCF7 breast cancer cells cultured with (A) Medium control and (B) MTX control (50 µg/mL). The percentages of live, apoptotic, and dead cells were calculated from FL1/FL3. Annexin-V-FITC was used to detect apoptotic cells, and propidium iodide (PI) was used to detect dead cells. J1~ necrotic cells, J2 ~ dead cells, J3 ~ live cells, and J4 ~ apoptotic cell. 15

Fig. S15. Fluorescence microscopy analysis of apoptotic and dead cells in MCF7 breast cancer cells cultured with (A) MTX-DMSO as control. a, b, c, d, and e, represents the various concentrations of MTX ranging from 4.6 µg/mL, 11.5 µg/mL, 23 µg/mL, 36 µg/mL, and 50 µg/mL, respectively. (A stock solution of MTX was prepared by first dissolving MTX in 200 µL DMSO, and volume was made up to 10 mL with cell culture medium (DMEM). This stock solution was further diluted to get required concenration of MTX. (B) The percentage of live, apoptotic, and dead cells as

calculated by Infinity Analyze software (Infinity Analyze 3[R], Lumenera Corporation). Error bars represent standard deviation. n=3. Scale bars ~ 200 µm. 16

Fig. S16 Confocal laser scanning microscopic images of cellular uptake in MCF7 cells cultured with free Oregon-green. Nuclei of cells were stained with Hoechst (blue fluorescence) and cell membranes were stained with CellMask™ deep red plasma membrane stain (red fluorescence). 17

Fig. S17. Representative fluorescence microscopy image of MCF7 cells in apoptosis stage. Arrows showing bright blue condensed or fragmented chromatin, a characteristic feature of the cells entering apoptosis. 17

List of Supplementary Schemes

Scheme S1. Scheme for the synthesis of Oregon-green. 10

1. Measurements

1.1. Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were recorded on a Bruker 400 spectrometer at 399.8 MHz (^{1}H) and 100.5 MHz (^{13}C). All chemical shifts were recorded in ppm using deuterated solvents and referenced against tetramethylsilane (0 ppm). NMR spectra were analysed using MestRENova 6.0.2 software (Mestrelab Research S.L).

1.2. Fourier transform infrared (FTIR) spectroscopy

FTIR spectra were recorded on an Agilent Technologies Cary 630 FTIR spectrophotometer fitted with a diamond single reflection ATR unit. All spectra were acquired by directly placing a small amount of a dried sample on a clean crystal of the instrument and were referenced against background spectra obtained by scanning the clean crystal before addition of the sample. Transmittances were recorded in the range 4000-650 cm^{-1} and data were processed using the Agilent MicroLab software suite.

1.3. Size exclusion chromatography (SEC)

SEC was performed on a Polymer Laboratories GPC 50 equipped with a differential refractive index detector. The mobile phase was HPLC grade $CHCl_3$ at 30 °C and a flow rate of 1 mL/min. Separations were performed on a pair of PLgel Mixed-D columns (300 × 7.8 mm, 5 µm bead size, Polymer Labs UK) fitted with a matching guard column (50 × 7.8 mm). The number average molecular weight (M_n), weight average molecular weight (M_w), and poly dispersity were calculated based on a standard calibration method using

poly(styrene) narrow molecular weight standards in the range of 100 Da–500 kDa. Chromatographs were analysed using Polymer Labs Cirrus 3.0 software. Some samples were also analysed using HPLC grade tetrahydrofuran as eluent at 40 °C.

1.4. Ultraviolet-visible spectroscopy (UV-Vis)

All UV-Vis spectra were recorded on a Beckman Coulter DU 800 UV spectrophotometer using quartz cuvettes. A sample volume of 700 µL was used and all spectra were referenced against a background of pure DMSO.

1.5. Fluorescence spectroscopy

Fluorescence spectra were recorded using an Agilent Cary Eclipse fluorescence spectrophotometer. The fluorescence intensities were measured against appropriate blank solutions at room temperature using a quartz cuvette. The excitation and emission slit width of 5 nm was selected for all measurements.

1.6. Dynamic Light Scattering (DLS)

The size distributions of micelles were measured by dynamic light scattering (DLS) using a Malvern Zetasizer (Nano-ZS, Malvern Instruments Ltd., UK). Samples were prepared in Milli-Q water and measurements were recorded at 25 °C and at a 173-degree angle as aqueous suspensions in polystyrene disposable cuvettes. Samples were illuminated using 633 nm wavelength (4mW) laser and data were analysed using Malvern ZetaSizer software version 7.11.

1.7. Zeta potential measurement

Zeta (ζ-) potentials measurements were derived from electrophoretic mobilities determined using a Zetasizer Nano ZS (Malvern Instruments Ltd., UK). Three measurements of each sample with at least 10 repeat data capture points each were carried out. Mean and standard deviations of ζ-potentials were calculated.

1.8. Transmission electron microscopy (TEM)

The morphologies of micelles were characterized by a Tecnai G2 (FEI, Oregon, USA) transmission electron microscope at electron voltages of 100 KV. TEM samples were prepared by placing a drop of micellar solution onto Formvar® coated copper grids and the suspensions were allowed to settle for few minutes. The excess of solution was removed with a filter paper and the samples allowed to air dry prior to analysis.

1.9. Electrospray ionization mass spectrometry

Electrospray ionization mass spectrometry was performed on a Micromass LCT KC453 spectrometer. Mass spectra were recorded using electrospray (ES+) or (ES-). Samples were prepared as diluted solution in acetonitrile or HPLC grade methanol.

1.10. Confocal laser scanning microscopy

The cellular uptake of micelles was observed using a Zeiss 510 Meta Confocal microscope. A 488 nm wavelength laser was used to excite the Oregon-green (excitation/emission maxima = 495/521 nm) and CellMask™ deep red plasma membrane stain (excitation/emission maxima = 649/666) was excited with a laser of 633 nm wavelength. The Hoechst 33342 stain (excitation/emission maxima = 350/461) was illuminated with a 100-watt high-pressure mercury plasma arc-discharge lamp (HBO 100). The control untreated cells (without micelles) were used to subtract the background or auto fluorescence.

2. Supplementary Methods

2.1. Synthesis of Oregon-green

4-Fluororesorcinol (500 mg, 3.90 mmol) was dissolved in 3 mL methanesulfonic acid. The system was deoxygenated using nitrogen gas for 30 minutes before adding trimellitic anhydride (394 mg, 1.95 mmol). Afterwards, the reaction was stirred for 48 h at 80 °C and was then allowed to cool down and poured into 7 volumes of cold water. The resultant precipitate was filtered and dried using rotary evaporator. Finally, the solid was dissolved in a small amount of methanol and precipitated again in cold water. The solid was subsequently dried using a rotary evaporator, and the final product was characterized by ^1H NMR and electrospray ionization mass spectrometry. ^1H NMR (400 MHz, CD_3OD) δ (ppm): 8.38 (s, 1H), 8.20 (d, 1H), 7.40 (d, 1H), 6.85 (s,s, 2H), 6.50 (s,s 2H).

2.2. Critical micelle concentration (CMC)

The critical micelle concentration (CMC) of block copolymers was measured by fluorescence spectroscopy using pyrene as a probe. To estimate CMC, a stock solution of pyrene was prepared in acetone and a known volume was transferred into a number of vials to obtain 6×10^{-7} M of pyrene in each vial. The vials were then placed in the dark for four hours at room temperature in a fume cupboard to allow evaporation of acetone. Block copolymer solutions ranging from 0.1-100 µg/mL were added into the pyrene-containing

vials. The vials containing block copolymer solution and pyrene were then agitated overnight (in dark). Fluorescence spectra of solutions were recorded in the range of 350 to 450 nm after excitation at 335 nm using an Agilent Cary Eclipse fluorescence spectrophotometer. The slit width was set at 5 nm for both excitation and emission. The ratio of the peak intensities at 373 and 383 nm (I_1/I_3) was plotted against the logarithm of polymer concentration to find the inflection point. The corresponding concentration at this point was considered to be the polymer CMC.

2.3. Cell culture

Human breast cancer cells (MCF7) were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% L-glutamine at 37 °C with 5 % CO_2. Cells were split after reaching 90 % confluency using fresh cell culture medium. Trypsin/EDTA was used to detach the cells that were adhered to tissue culture flask. Cell counting was performed using a haemocytometer.

3. Supplementary Figures

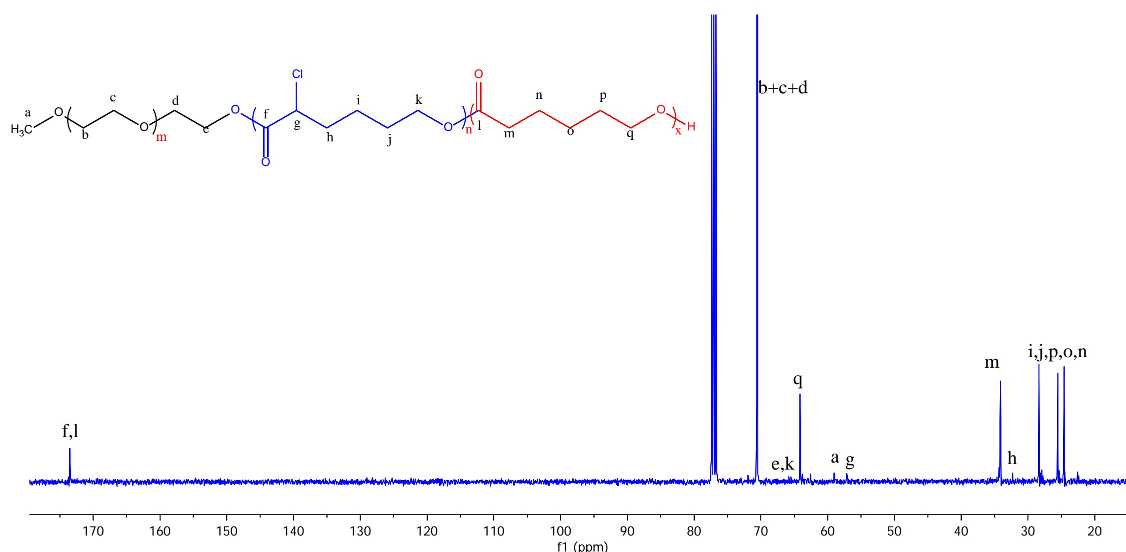


Fig. S1. ^{13}C NMR spectrum of mPEG-*b*-poly(εCL-*co*-αClεCL) in $CDCl_3$.

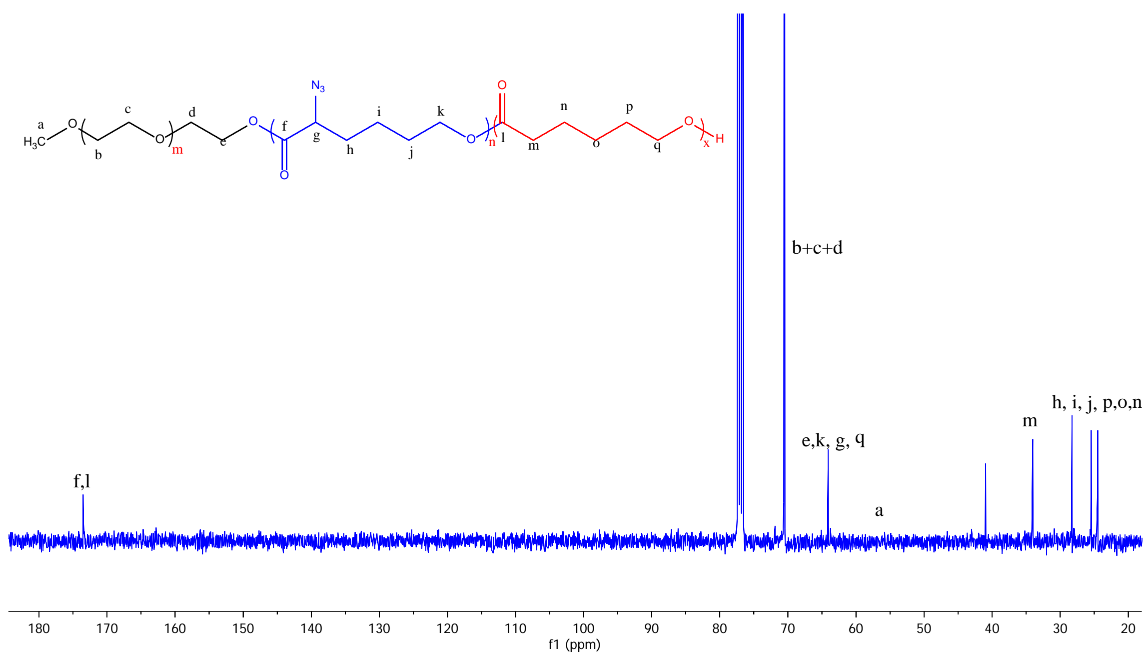


Fig. S2. ^{13}C NMR spectrum of mPEG-*b*-poly(εCL-*co*-αN_3CL) in $CDCl_3$.

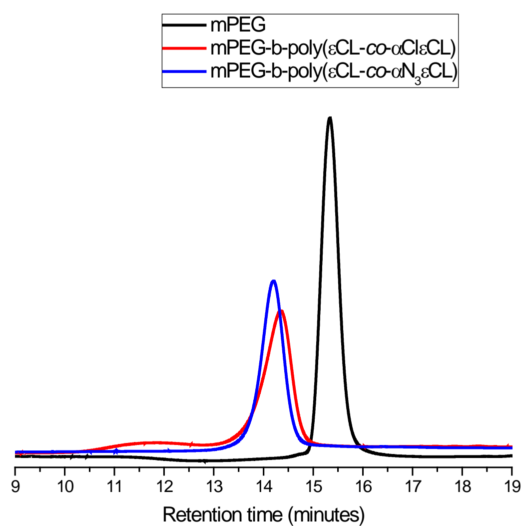


Fig. S3. SEC traces of mPEG, mPEG-*b*-poly(εCL-*co*-αClεCL), and mPEG-*b*-poly(εCL-*co*-αN_3CL) in $CHCl_3$.

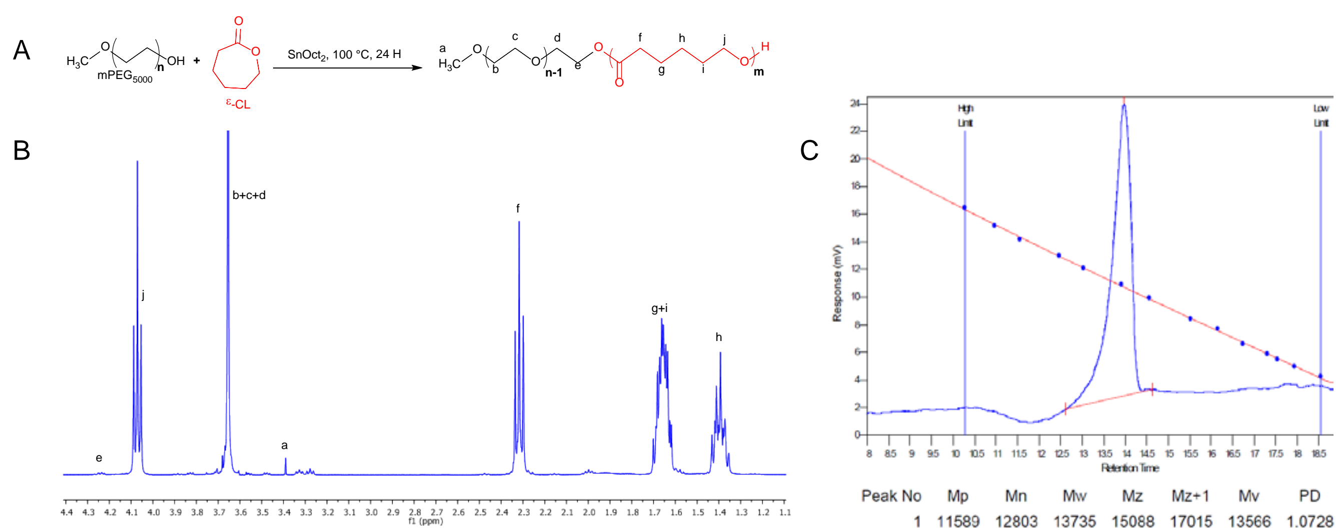


Fig.S4 (A) Schematic representation for the synthesis of mPEG-PCL, (B) 1H NMR spectrum, and (C) SEC traces in CHCl3 (PS calibration).

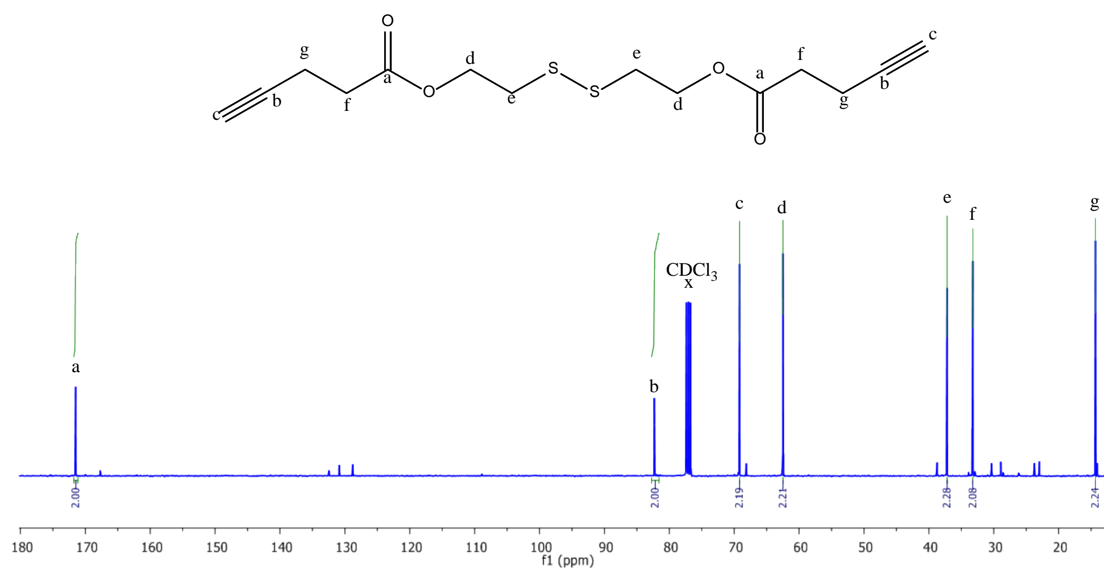


Fig. S5. ^{13}C NMR spectrum of redox-responsive bis-alkyne-ethyl disulfide crosslinker.

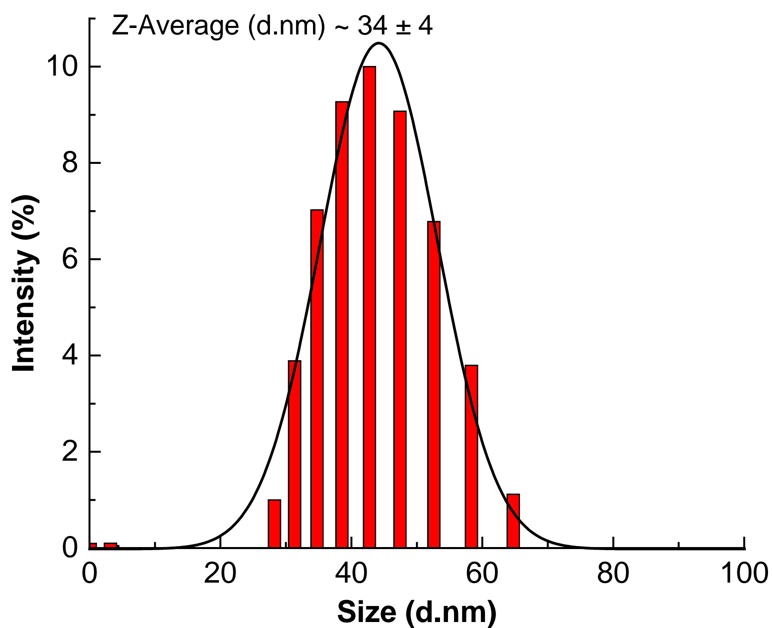
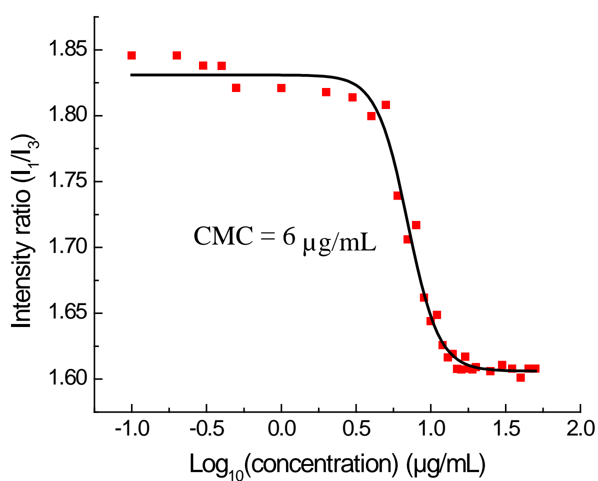


Fig. S6 Size distribution of mPEG-PCL micelles measured by DLS.



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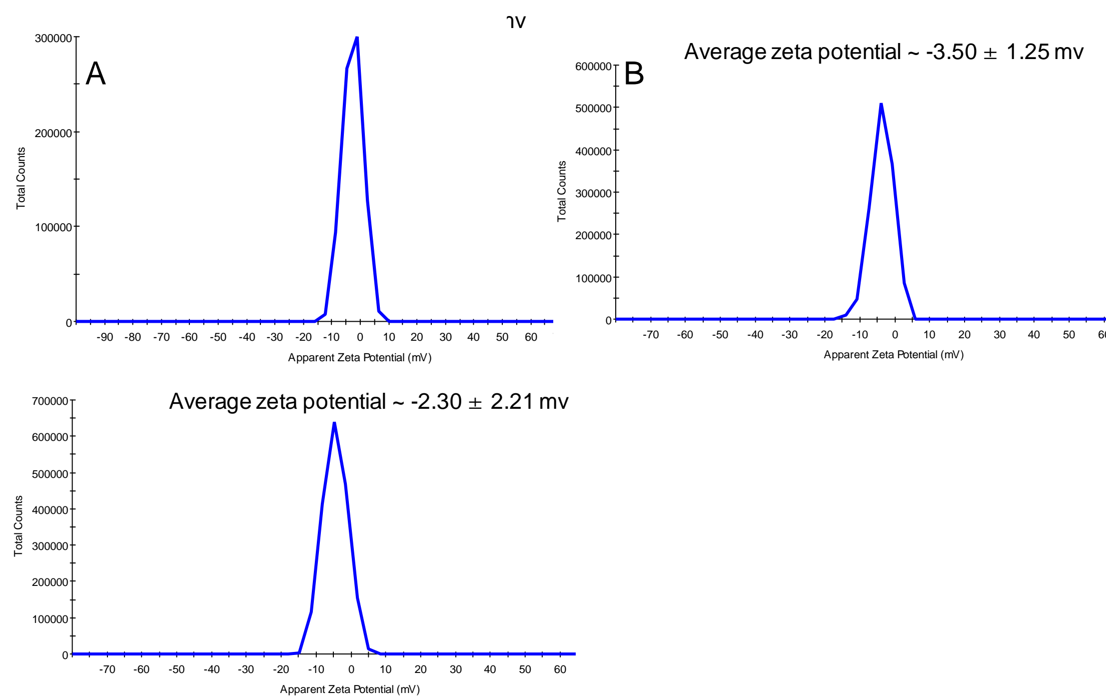

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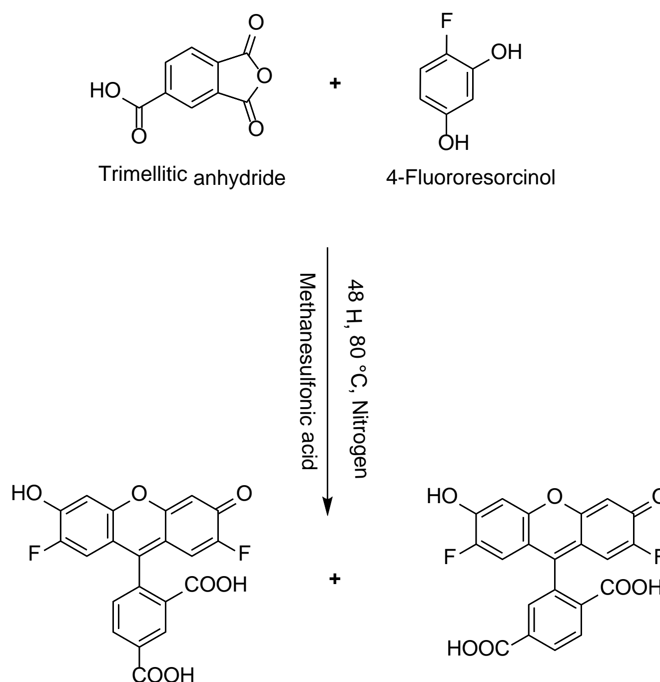


Scheme S1. Scheme for the synthesis of Oregon-green.

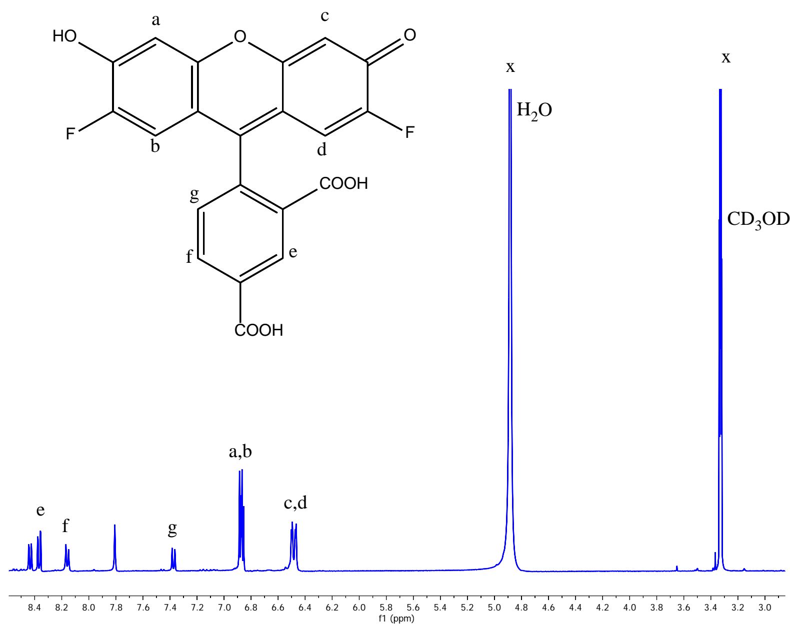


Fig. S9. ^{1}H NMR spectrum of Oregon-green.

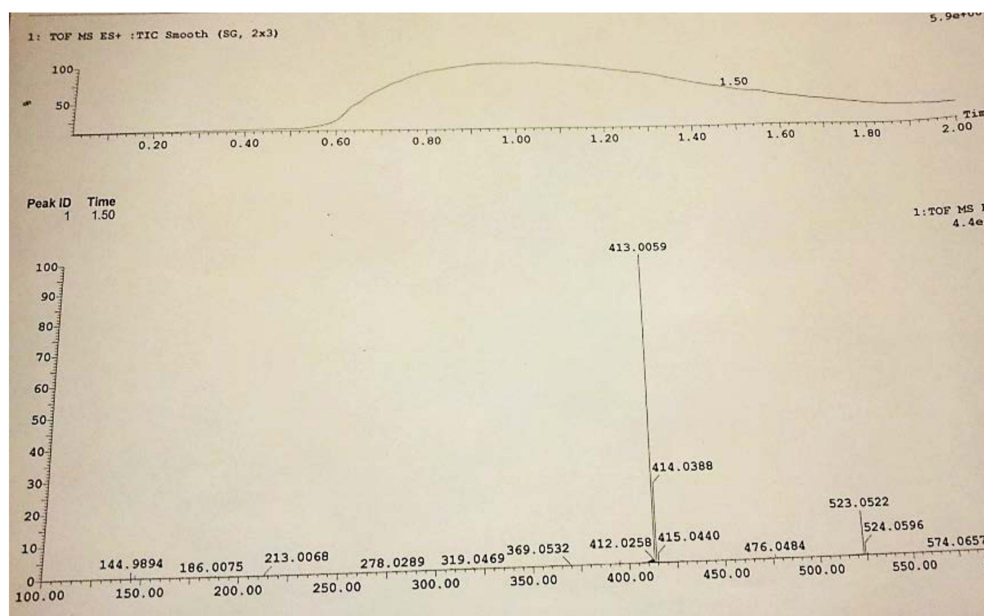
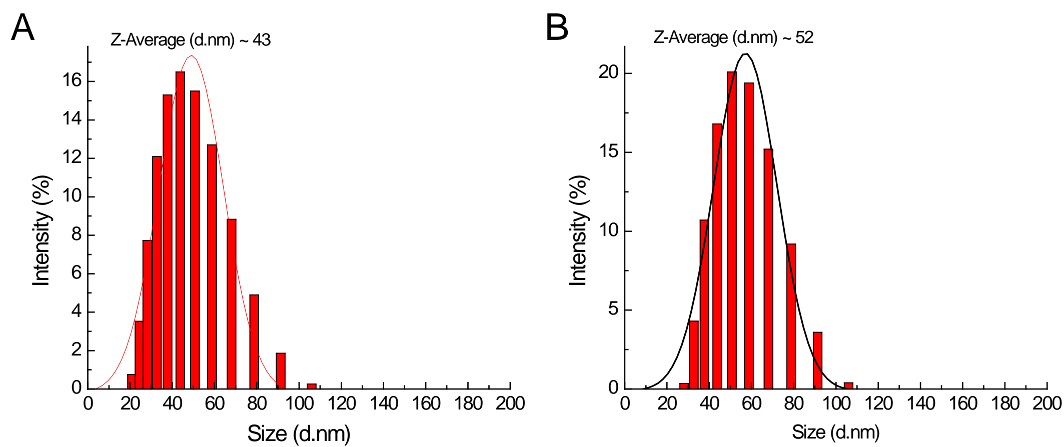


Fig. S10. Electrospray ionization mass spectrum of Oregon-green.



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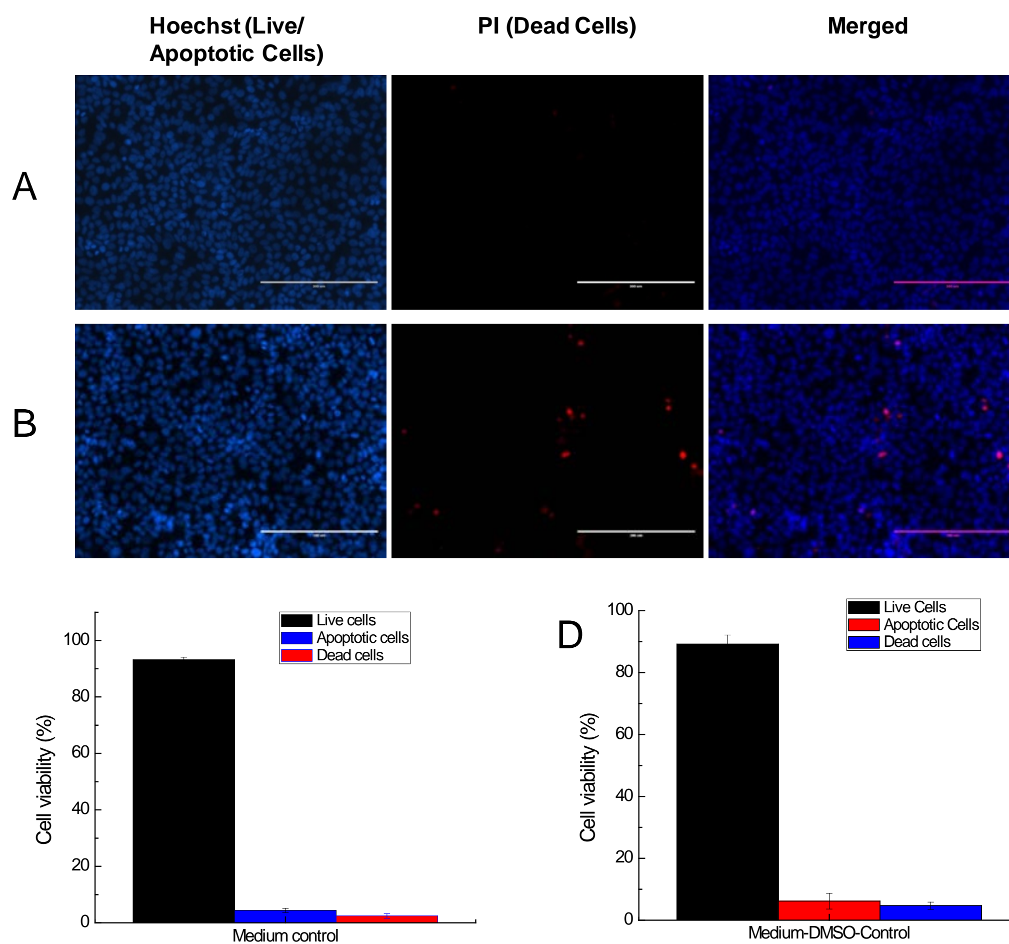

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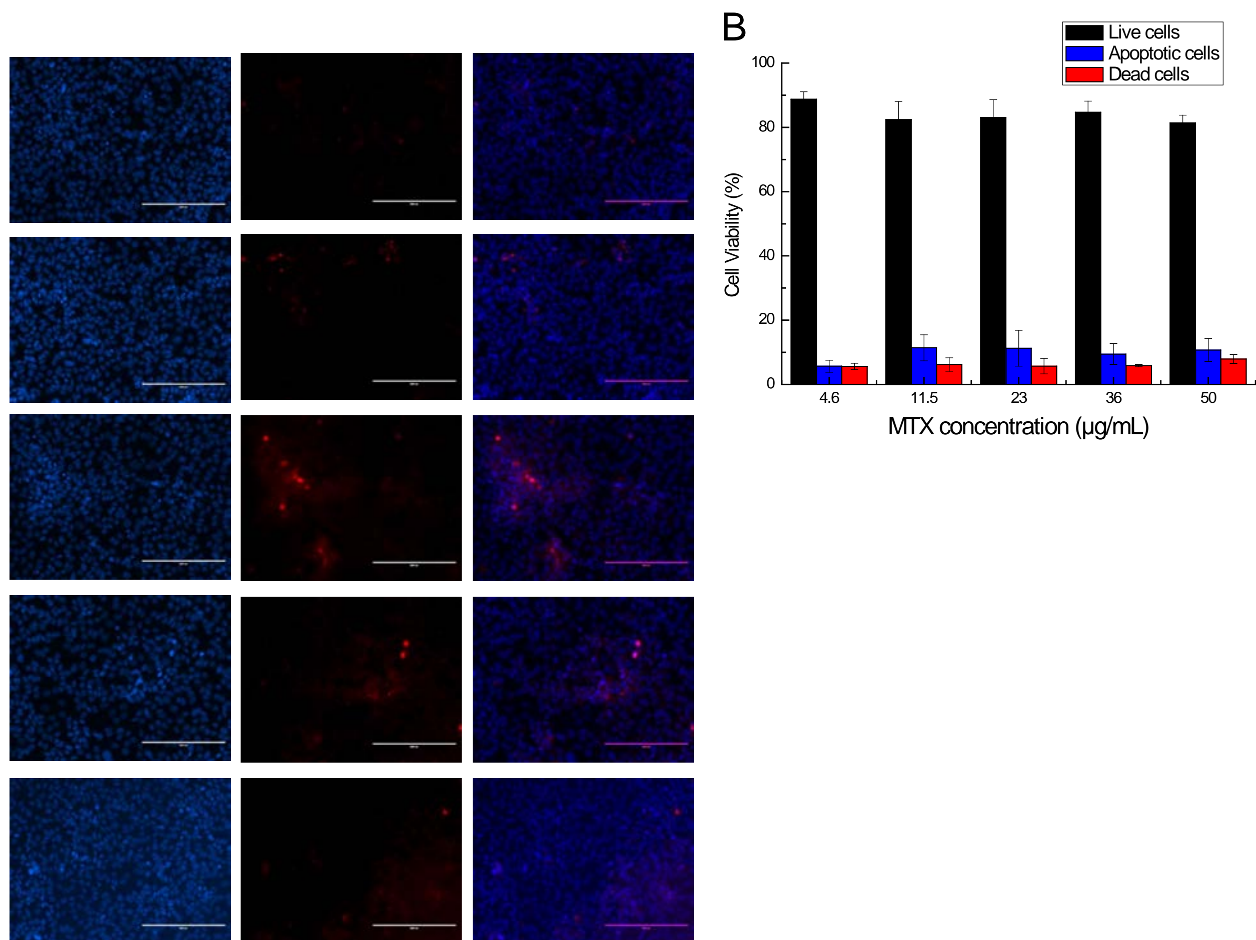

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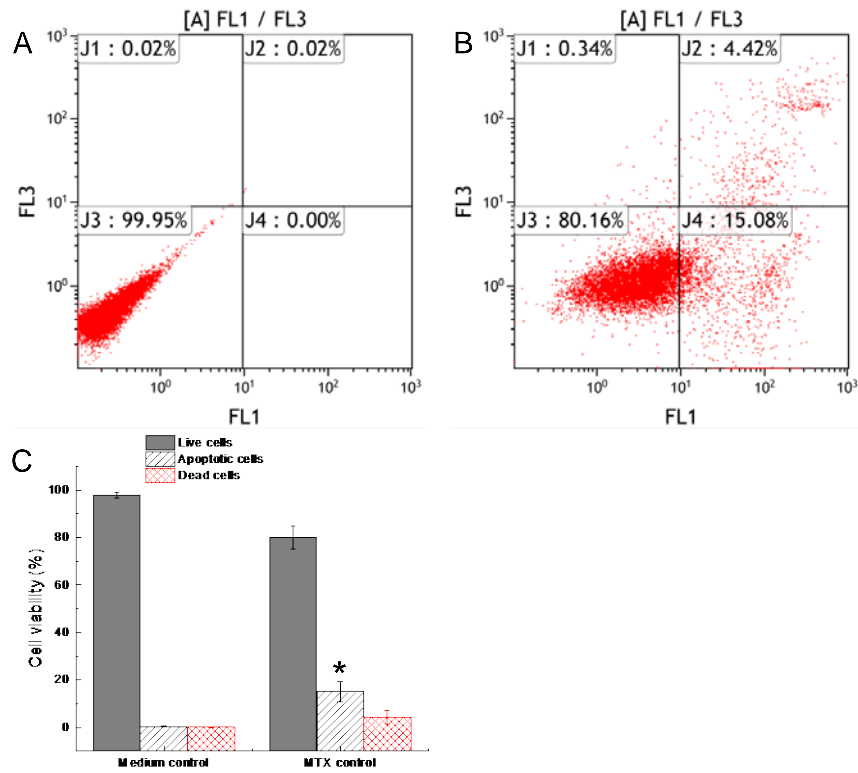

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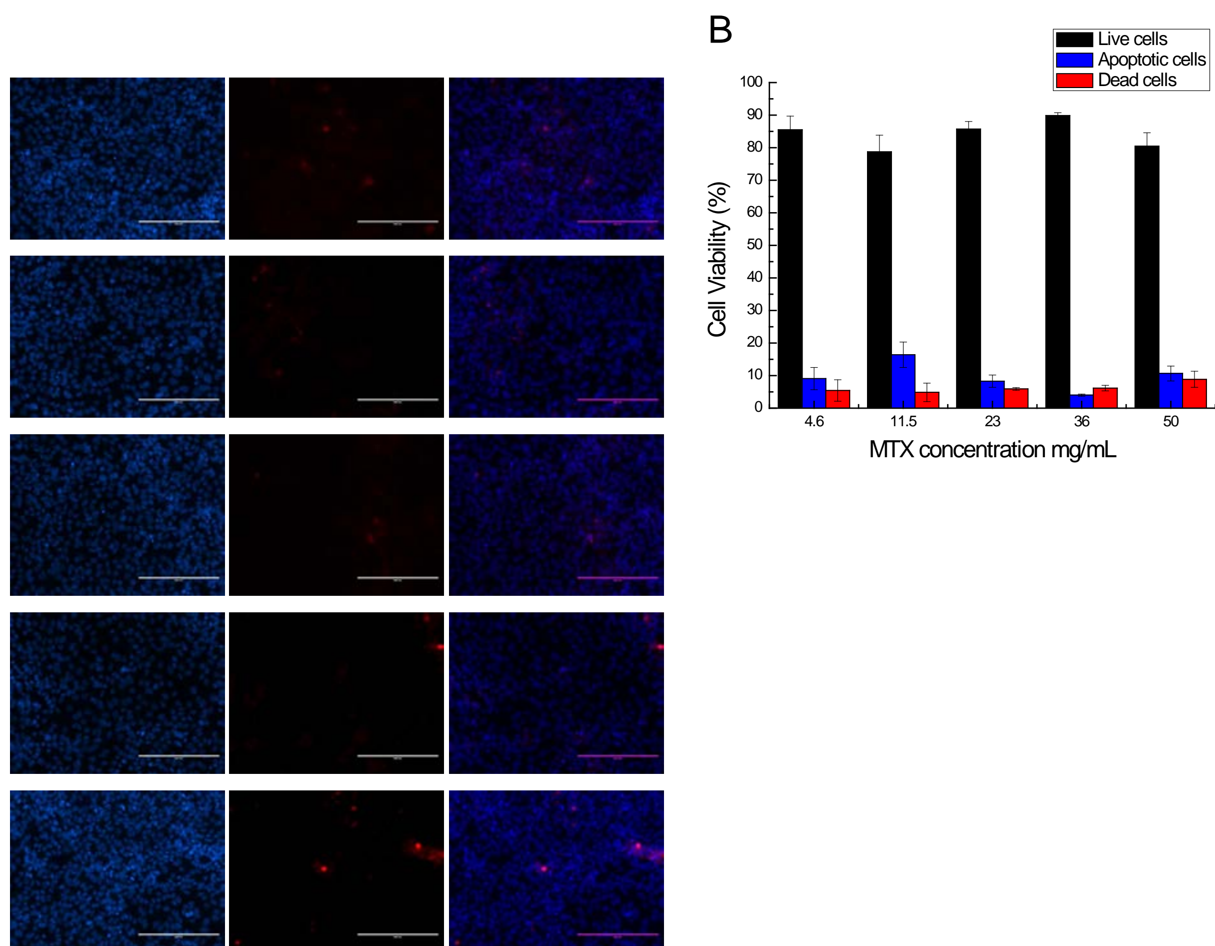

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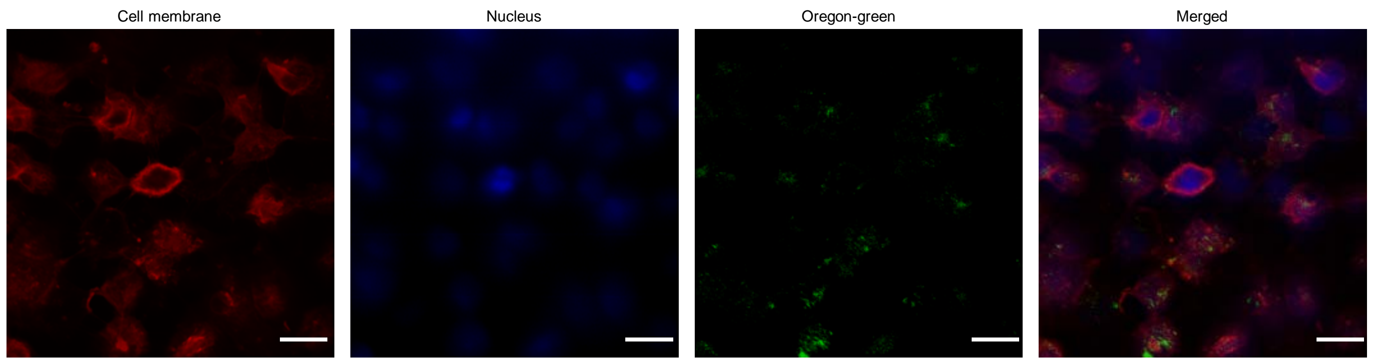
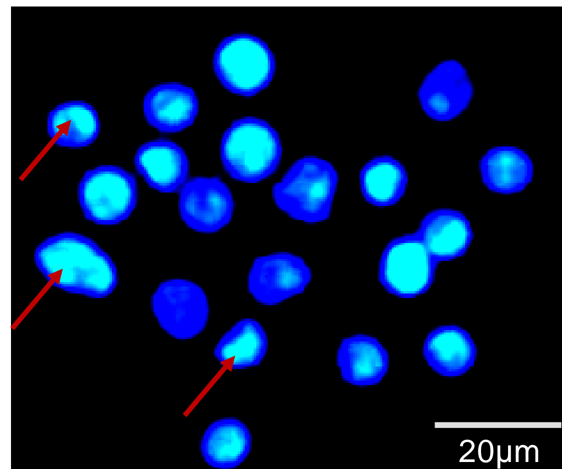


Fig. S16 Confocal laser scanning microscopic images of cellular uptake in MCF7 cells cultured with free Oregon-green. Nuclei of cells were stained with Hoechst (blue fluorescence) and cell membranes were stained with CellMask™ deep red plasma membrane stain (red fluorescence). Scale bars~ 20 µm.



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