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Mechanistic Insight into the Singlet Oxygen-Triggered Disassembly of Hypoxia-Responsive Polymeric Micelles

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Experimental Section

Materials

L-Aspartic acid *β*-benzyl ester (BLA) was purchased from Beijing HWRK Chem Co., Ltd. (Beijing, China). Nitroimidazole (i.e. 2-Nitroimidazole) (NI) and Chlorin e6 (Ce6) were purchased from Beijing J&K Scientific Co., Ltd. (Beijing, China). *N*-(3-bromopropyl)phthalimide was purchased from Aladdin Industrial Corporation (Shanghai, China). Methoxy-polyethylene glycol amine (mPEG-NH_2, 5000 Da) was sourced from Shanghai Pengshuo Technology Co., Ltd. (Shanghai, China). Potassium carbonate (K_2CO_3), N,N-dimethylformamide (DMF), diethyl ether, hexane, dimethyl sulfoxide (DMSO), chloroform ($CHCl_3$), hydrochloric acid (HCl), trifluoroacetic acid (TFA), acetone, disodium hydrogen phosphate (Na_2HPO_4) and sodium hydroxide (NaOH) were obtained from Guangfu Fine Chemical Research Institute (Tianjin, China). Deuterium dimethyl sulfoxide (DMSO-d_6), deuterium dimethyl sulfoxide and deuterium oxide were purchased from Jinouxiang Science & Technology Co., Ltd. (Beijing, China). Acetonitrile (ACN), tetrahydrofuran (THF), methanol and absolute ethanol were obtained from Tianjin Concord Technology Co., Ltd. (Tianjin, China). 1-hydroxybenzotriazole (HOBT) was purchased from Bide Pharmatech Ltd. (Shanghai, China). Triphosgene (BTC) was purchased from Sigma-Aldrich (Beijing, China). *N*,*N*-diisopropylethylamine (DIPEA) was purchased from Shanghai Taitan Technology Co., Ltd. (Shanghai, China). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC.HCl) was purchased from Shanghai Medpep Co., Ltd. (Shanghai, China).

Instruments

Nuclear magnetic resonance analysis (^{1}H NMR and ^{13}C NMR) was carried out on a Bruker AVANCE Ⅲ (400 MHz or 600 MHz) NMR spectrometer with deuterated chloroform, deuterium oxide and deuterium dimethyl sulfoxide as the solvent. The hydrodynamic diameter and zeta potential were analyzed at 25°C by Zetasizer Nano ZS (Malvern Instrument Ltd.) and the scattering angle was set at 173°. The micelle morphology was observed using a JEM-2100F transmission electron microscope (TEM). The fluorescence analysis was conducted by a FLS980 fluorescence spectrometer (Edinburgh Instruments Ltd.) and a microplate reader (Tecan Group Ltd.) High resolution mass spectrometry (HRMS) analysis was performed on a Bruker quadrupole time-of-flight mass spectrometer at 25°C; the mobile phase was acetonitrile (0.4 mL/min) and the injection volume was 0.2 μL.

Synthesis of the nitroimidazole derivatives

The synthesis of the nitroimidazole derivative, 3-(2-nitro-1H-imidazol-1-yl)propan-1-amine (NI-NH_2) utilized a previously published method with slight modification (Scheme S1)[1]. In brief, *N*-(3-bromopropyl) phthalimide (2.5 g, 9.0 mmol), potassium carbonate (1.3 g, 9.0 mmol), and NI (1.0 g, 9.0 mmol) were dissolved in 25 mL DMF. The mixture was maintained at 110°C for 2 h, followed by solvent removal by rotary-evaporation. The obtained solid was washed with double-distilled water in triplicate to get the intermediate product, i.e. 2-(3-(2-nitro-1H-imidazol-1-yl)propyl)isoindoline-1,3-dione (yield: 94%). Then the intermediate (2.1 g, 7.0 mmol) was dissolved in ethanol (50 mL), followed by hydrazine supplementation (0.6 mL, 13.0 mmol) and reflux at 80°C for 2 h. After being cooled to 0°C, the reactants were filtered and the filtrate was rotary-evaporated to get a yellow liquid. The obtained liquid was dissolved in 30 mL HCl aqueous solution (1 M), followed by filtration, solvent removal, and re-crystallization in methanol- ethyl acetate system. The final product (i.e. NI-NH_2·HCl) was 3-(2-nitro-1H-imidazol-1-yl)propan-1-amine hydrochloride (yield: 71%). The obtained NI-NH_2 and corresponding intermediates were characterized in terms of ultraviolet (UV) spectroscopy, proton/carbon nuclear magnetic resonance spectroscopy (^{1}H NMR, ^{13}C NMR) and mass spectroscopy (MS).

Preparation of polymer-nitroimidazole conjugates

The synthesis of mPEG-PBLA and mPEG-PAsp employed previously published protocols (Scheme S2).[2,3] In brief, *L*-Aspartic acid *β*-benzyl ester (5 g, 22.4 mmol) and triphosgene (3.3 g, 11.1 mmol) reacted in 50 mL THF at 50°C for 3 h, followed by crystallization (THF/hexane) to get the monomer (BLA-NCA) (yield: 82%). Then, BLA-NCA (1.5 g, 6.0 mmol) was polymerized at 35°C under nitrogen atmosphere with the aid of mPEG-NH_2 (1.0 g, 0.2 mmol) as the initiator. After 48 h, mPEG-PBLA was obtained by precipitating the reactants in diethyl ether, followed by purification by dialysis against water and lyophilization (yield: 71%). Then, mPEG-PBLA (1.0 g, 0.01 mmol) was treated by sodium hydroxide aqueous solution (0.5 M, 13.2 mL) at 30°C for 24, followed by adjusting pH to 2-3 using hydrochloride acid (0.1 M) and dialysis against water (molecular weight cut-off/MWCO: 1000 Da) (yield: 80%). The conjugation of nitroimidazole to the polymer backbone involves a typical amidation process.[2] Briefly, mPEG-PAsp (0.50 g, 0.07 mmol) dissolved in anhydrous DMF (5 mL) was activated by EDC (268.4 mg, 1.4 mmol) under nitrogen

protection, followed by the addition of HOBT (189.2 mg, 1.4 mmol) DMF solution (2 mL). After 1 h, the active polymer solution was dropwise added to the mixture of NI-NH_2·HCl (1.2 g, 5.6 mmol) and DIPEA (2.0 mL, 11.2 mmol) in DMF (50 mL). The reaction was maintained at 40°C for 48 h and then the system was dialyzed against water (MWCO: 1000 Da). The final product, polymer-nitroimidazole conjugate (mPEG-PAsp-NI) was obtained after lyophilization (yield: 72%). The obtained conjugate was also characterized by ^{1}H NMR. The UV absorption of mPEG-PAsp-NI as well as mPEG-PAsp was analyzed at an identical concentration of 190 μg/mL with methanol or water as the solvent. The molecular weight of mPEG-PAsp-NI and the corresponding mPEG-PAsp was determined by Waters gel permeation chromatography instrument coupled with a Waters 2414 refractive index detector. The elution was achieved by an Ultrastyragel column (10 μm, 7.8 mm x 30 cm) at 35°C with tetrahydrofuran as the mobile phase (1 mL/min) and polystyrene as the calibration standard.

Preparation and characterization of micelles

The preparation of polymer conjugate micelles utilized a typical dialysis method.[4] Briefly, mPEG-PAsp-NI or mPEG-PBLA (200 mg) and Ce6 (20 mg) was dissolved in 5 mL DMSO and the solvent was dialyzed (MWCO: 1000 Da) against water for 24 h. The excess drug was removed by centrifugation prior to lyophilization (n = 3). The Ce6 loading in responsive micelles (RM) and control micelles (CM) was determined by high performance liquid chromatography using a previously published method.[5] The hydrodynamic diameter and zeta potential of both micelles were analyzed. For particle size analysis, the micelle concentration in deionized water was fixed at 1 mg/mL, whereas the particles (CM and RM) with or without Ce6 were dispersed in KCl (1 mL) prior to determining the zeta potential. Diluted samples were placed on the surface of carbon-coated copper grids and dried in the air prior to image-recording.

Critical micelle concentration analysis

As a stability indicator, the critical micelle concentration (CMC) was determined with the help of a fluorescent probe, pyrene.[6] A series of mPEG-PAsp-NI micelle aqueous solutions (0.4-400 μg/mL) was spiked with pyrene to maintain a final probe concentration at 0.5 μM. After 24 h's incubation with light protection, the samples' emission spectra (350-450 nm) were recorded. The excitation wavelength was 333 nm with a slit width of 5 nm and optical path length of 1 cm. The band intensity of 384 nm with reference to 373 nm (i.e. I_{384}/I_{373}) was plotted against the logarithm of the micelle concentration and the CMC value was obtained from the flexion point of the curve (n = 3).

Drug release analysis

A static vertical type Franz cell (ca. 17 mL) was employed for analyzing Ce6 release from responsive micelles based on a previous report.[7] The RM micelles (10 mg) were dispersed in 2 mL phosphate buffered saline (PBS, pH 7.4, 180 mM). The receiver fluid was also the same PBS containing a magnetic flea; the donor and receiver compartment was separated by a regenerated cellulose membrane (MWCO: 1000 Da). The temperature was controlled at 37°C using a thermostat water bath. The test was performed with or without light irradiation (laser wavelength: 660 nm; intensity: 100 mW/cm^2; duration: 10 min; position: 20 cm above the donor compartment). At pre-determined time points (0.5-36 h), 0.5 mL receiver fluid was withdrawn for drug quantification by HPLC and the same amount of fresh medium was supplemented. The data were presented as the cumulatively released Ce6 against time.

Cellular uptake and Cell viability assay

Murine breast cancer cells (4T1 cell) were obtained from the State Key Laboratory of Medicinal Chemical Biology, Nankai University. The culture medium was RPMI 1640 (Gibco, NY, USA) containing 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were seeded in 96-well plates at a density of 4×10^3 cells/well with the culturing conditions of 37°C with 5% CO_2. After 24 h, four formulations were applied including RM/Ce6 micelles, CM/Ce6 micelles, free Ce6, and placebo RM micelles. The dose of Ce6 ranged from 1.0 μg/mL to 5.0 μg/mL. After 2 h's incubation, the cells were washed with PBS (pH 7.4, 10 mM) and fresh medium (100 μL) was added. Then light irradiation (660 nm, 100 mW/cm^2) was applied for different duration (0, 1, 5, and 10 min). After 24 h, the viability of cells was determined by the standard of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.[8] Regarding the placebo micelles, the polymer dose was set to match that of drug-loaded micelles (i.e. RM/Ce6). The cellular uptake of three formulations with a fixed Ce6 dose (4 μg/mL) was analyzed after 2 hours' incubation, followed by cell washing by PBS, nuclear staining and Ce6 imaging.[S2]

Mechanism of singlet oxygen-induced nitroimidazole conversion

To verify the structure change of nitroimidazole in response to singlet oxygen challenge, NI-NH_2·HCl (1.0 mg, 4.9 μmol) and Ce6 (10 mg, 16.7 μmol) dissolved in 2 mL methanol were irradiated for 30 min (660 nm, 100 mW/cm^2), followed by a high-resolution mass spectrometry analysis at 25°C using a micrOTOF-Q II system. Apart from the mass analysis, ^{1}H NMR was also employed to monitor the structure change of nitroimidazole moiety. In brief, either NI-NH_2·HCl (1.0 mg, 4.9 μmol) or mPEG-PAsp-NI (1.0 mg, 0.13 μmol) were mixed with different amount of Ce6 (0, 5, 10, and 15 mg) in methanol (1 mL) followed by laser treatment (30 min, 660 nm, 100 mW/cm^2) prior to NMR analysis. The formation of oxamic aldehyde was confirmed using a fluorescent method (Scheme S3).[9] The mPEG-PAsp-NI (1.0 mg, 0.13 μmol) and Ce6 (1 mg) in methanol (1 mL) was irradiated under the same condition (30 min, 660 nm, 100 mW/cm^2). Afterwards, 800 μL 2,2'-furil (8.0 mM) solution in methanol and 300 μL ammonium acetate (3.0 M) solution in glacial acetic acid were added. The mixture was maintained at 90°C for 30 min prior to recording the emission spectrum by a microplate reader (E_x = 250 nm). The system without laser irradiation was used as control. The singlet oxygen-induced structure modification of nitroimidazole was further indirectly examined by particle analysis. The Ce6-loaded responsive micelle aqueous solution (1 mg/mL) was subject to radiation (660 nm, 100 mW/cm^2) for different duration (0, 5, 20 and 50 min) and the hydrodynamic and TEM diameters were tested 24 hours later.

References

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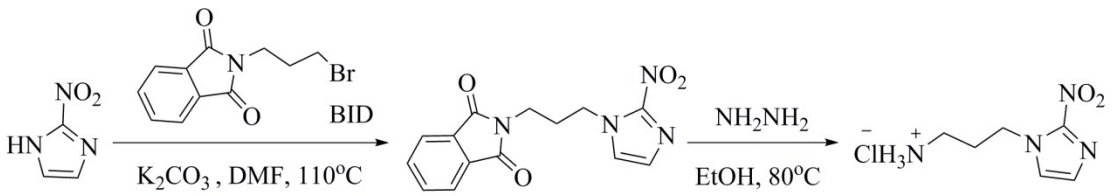
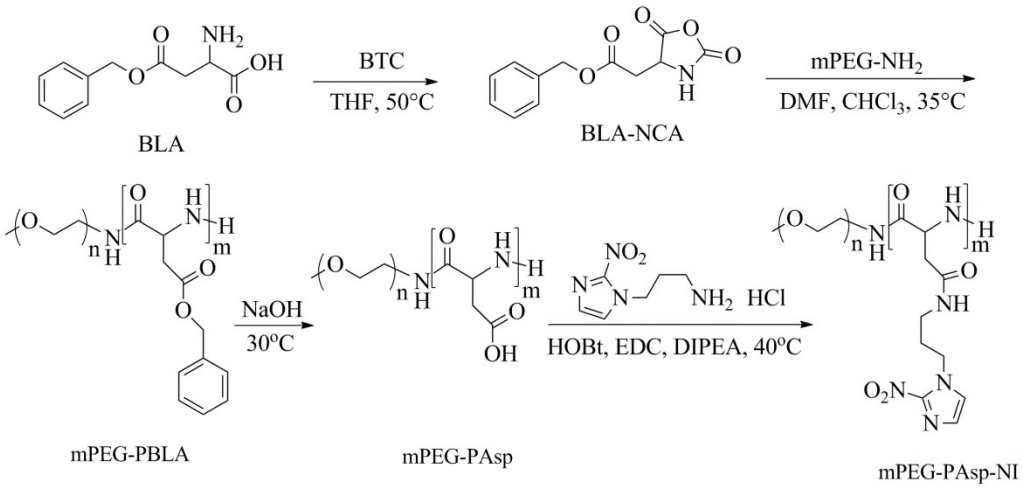
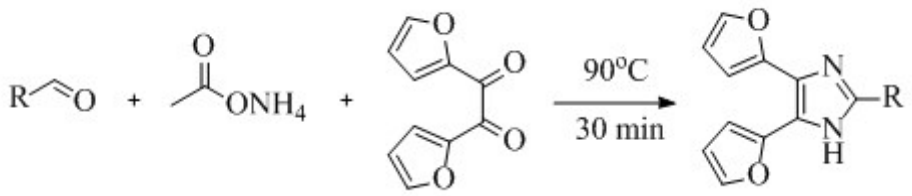


Scheme S1. Synthetic route of nitroimidazole derivative (NI-NH_2·HCl).



Scheme S2. Synthetic route of singlet oxygen-responsive polymer (mPEG-PAsp-NI) and the control polymer (mPEG-PBLA). mPEG, PBLA, PAsp, and NI represents methoxyl poly(ethyl glycol), poly(*β*-benzyl-*L*-aspartate), poly(aspartic acid), and nitroimidazole, respectively.



Scheme S3. The reaction scheme of aldehydes with 2,2' Furil to produce a fluorescence end-product.

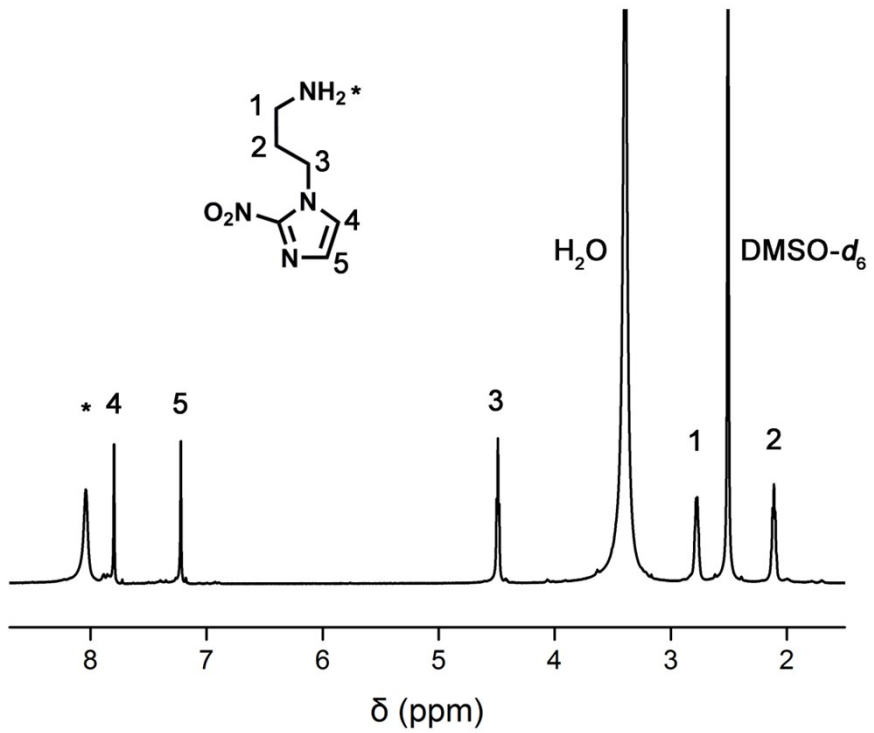


Figure S1. ^{1}H NMR Spectrum of NI-NH_2 (DMSO-d_6)

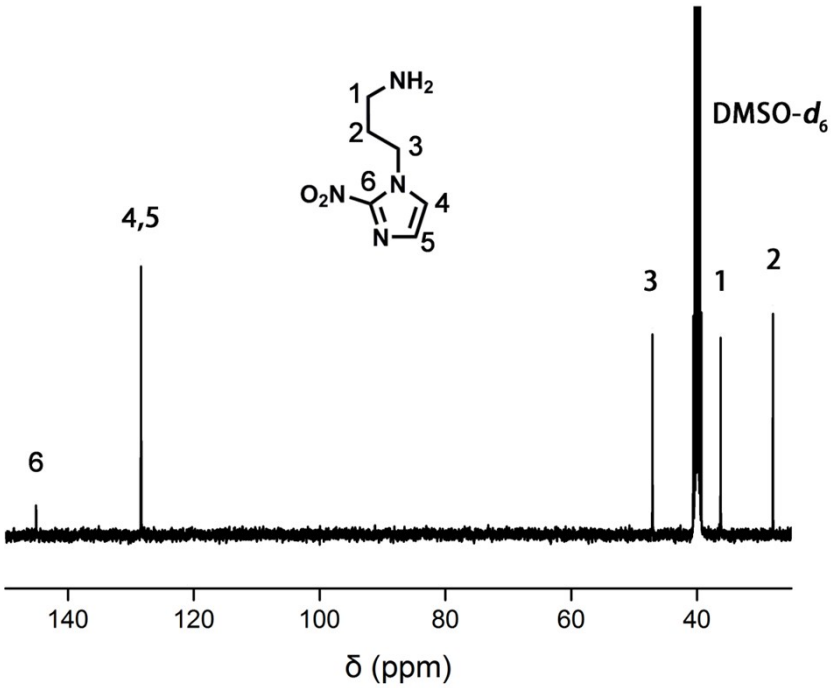


Figure S2. ^{13}C NMR spectrum of NI-NH_2. (DMSO-d_6)

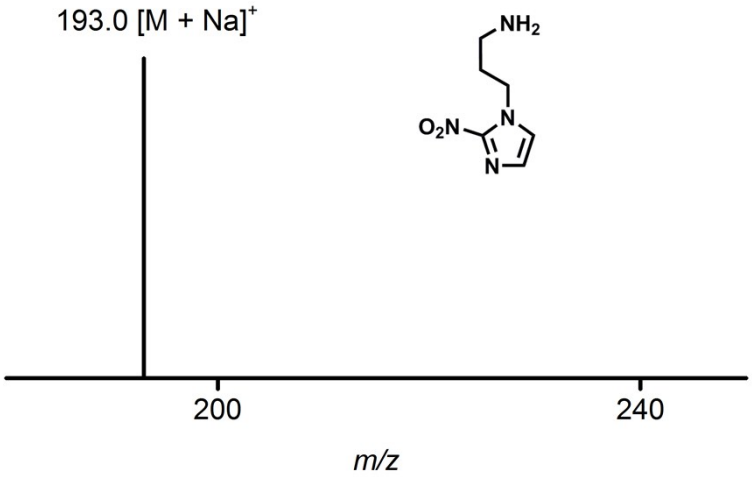


Figure S3. MS spectrum of NI-NH_2.

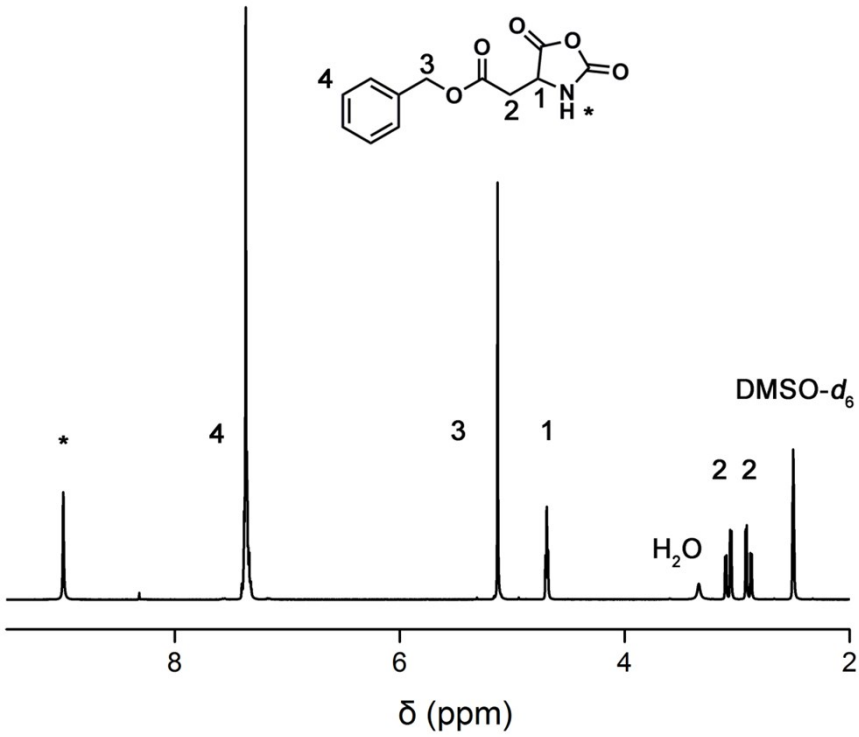


Figure S4. ^{1}H NMR spectrum of BLA-NCA (DMSO-d_6)..

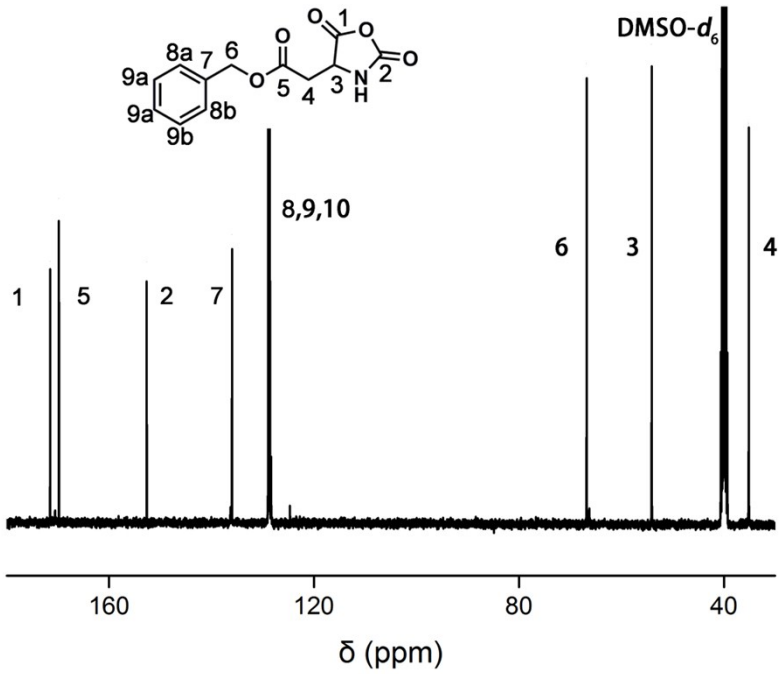


Figure S5. ^{13}C NMR spectrum of BLA-NCA (DMSO-d_6).

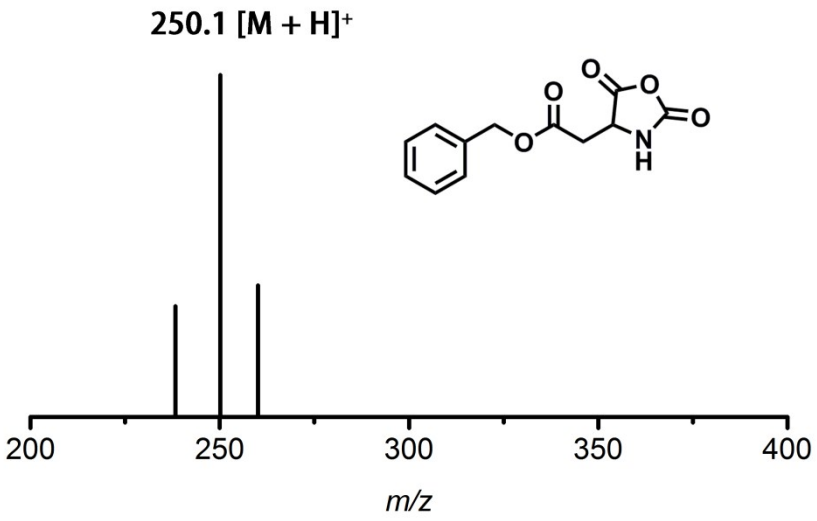


Figure S6. MS spectrum of BLA-NCA.

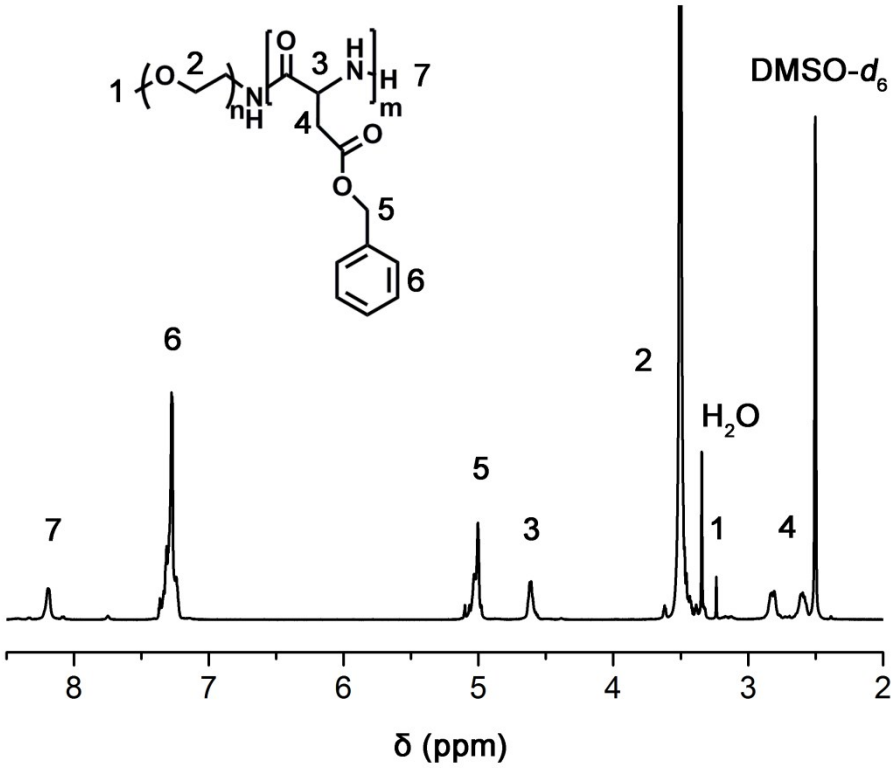


Figure S7 ^{1}H NMR spectrum of mPEG-PBLA (DMSO-d_6).

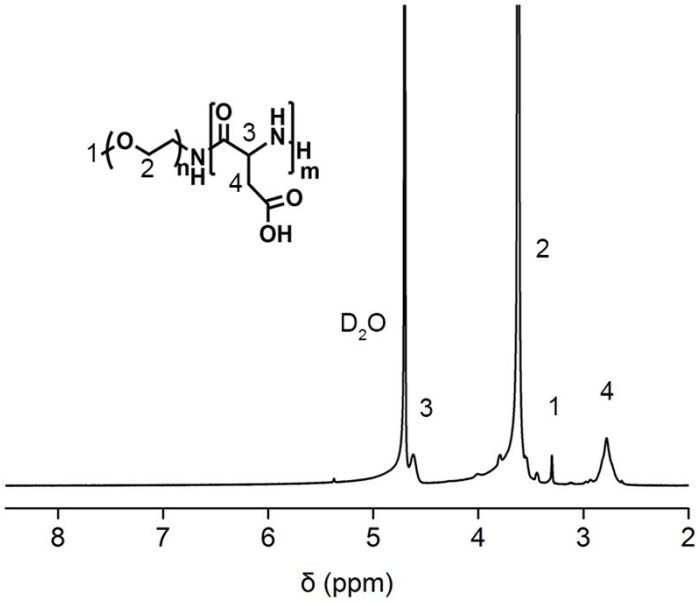


Figure S8. ^{1}H NMR spectrum of mPEG-PAsp (D_2O).

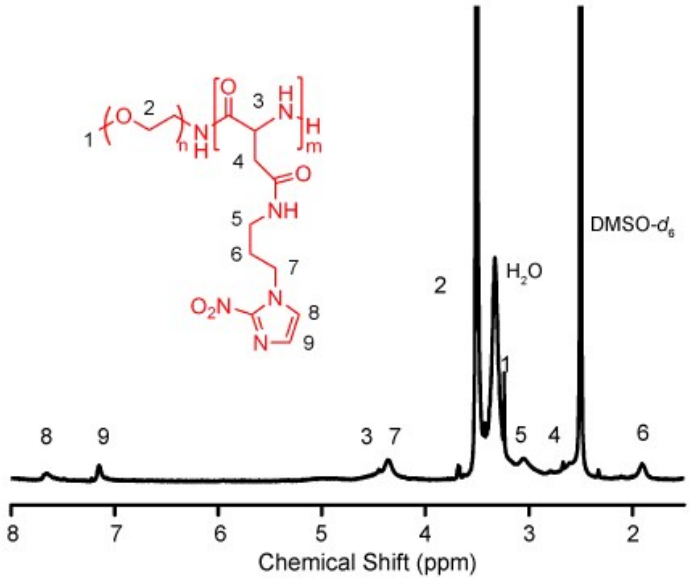


Figure S9. ^{1}H NMR spectrum of methoxy poly(ethylene glycol)-*co*-poly(aspartic acid)-nitroimidazole, i.e. mPEG-PAsp-NI (DMSO-d_6).

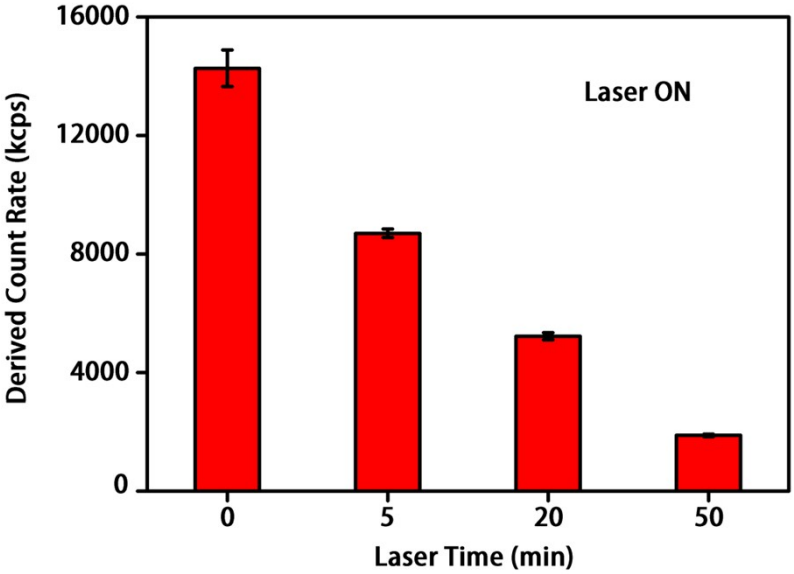


Figure S10. Derived count rate of micellar samples by dynamic light scattering upon laser treatment (0, 5, 20, 50 min), (660 nm, 100 mW/cm^2, n = 3).

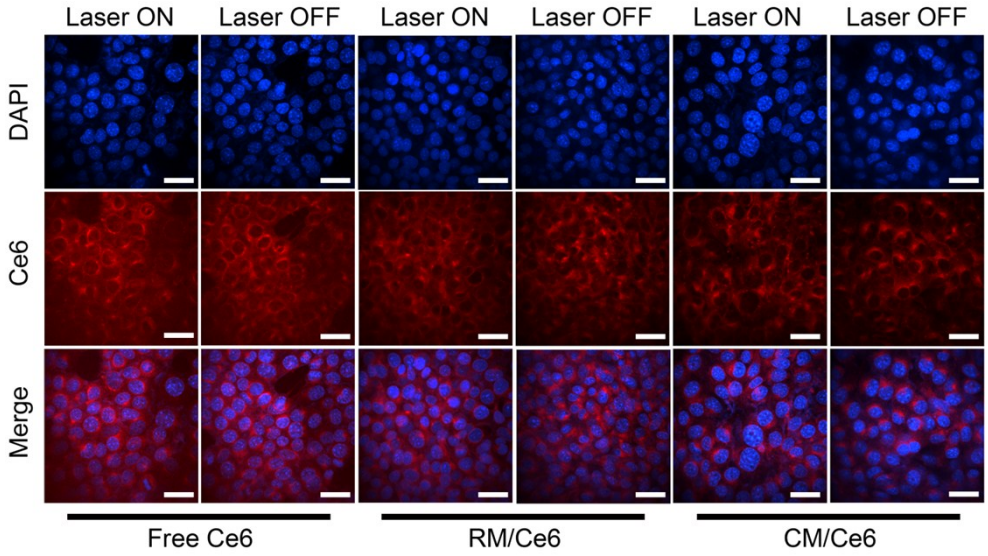


Figure S11. Internalization of three formulations (free Ce6, RM/Ce6, and CM/Ce6) in 4T1 cells post 2 hours' incubation. The Ce6 dose was fixed at 4 μg/mL for all three formulations. The "Laser ON" indicated that the cells received 10 min's laser irradiation (660 nm. 100 mW/cm^2) post sample incubation.

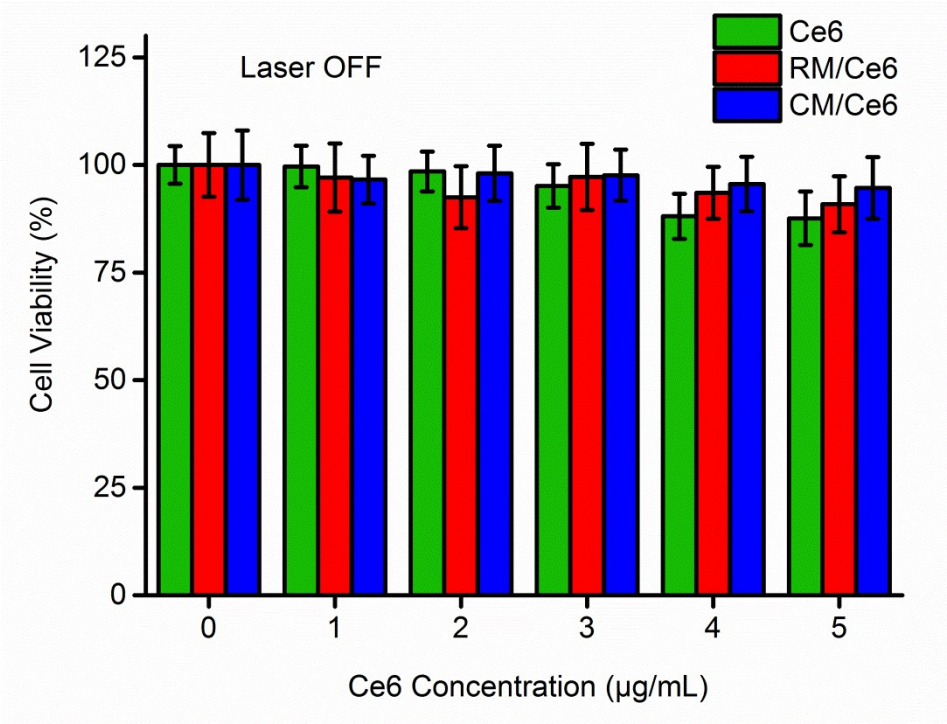


Figure S12. Cell viability of Ce6, RM/Ce6 and CM/Ce6 without light treatment (n = 4).

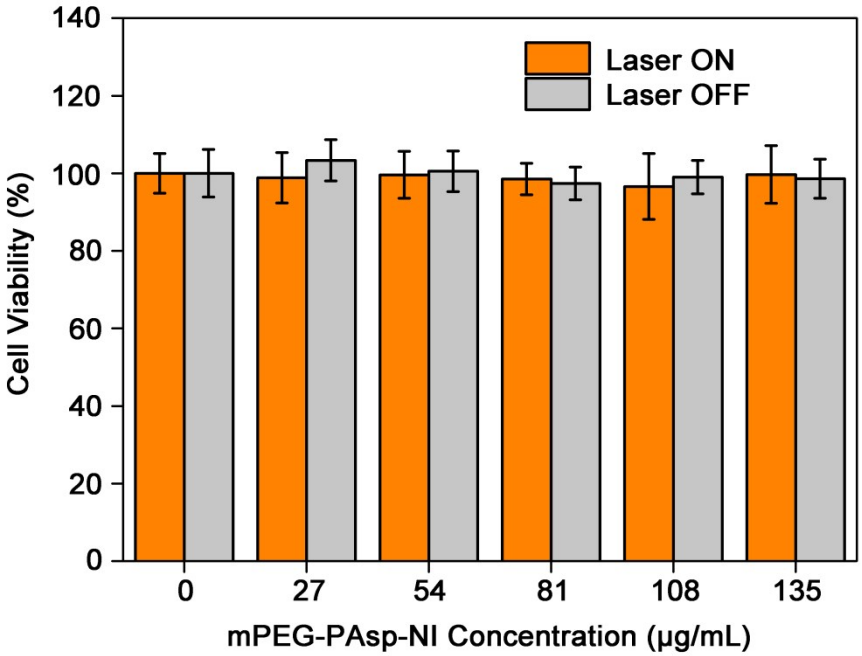


Figure S13. Cell viability of mPEG-PAsp-NI with/without light treatment for 10 min (660 nm, 100 mW/cm^2, n = 4).