Supporting Information

Dual-Labelled Polymeric Micelles for Singlet Oxygen Reporting in Biological Systems

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Experimental section

1. Materials

β-Benzyl-L-aspartate-*N*-carboxy anhydride (BLA-NCA) was purchased from Chuo kaseihin CO., INC (Tokyo, Japan). Dichloromethane (DCM), *N*, *N*-dimethylformamide (DMF) was purchased from Jujo synthetic chemistry labo. (Tokyo, Japan). Argon gas was purchased from Suzuki shokan CO., LTD. (Tokyo, Japan). Benzene, sodium hydroxide, lithium chloride, *N*-methyl-2-pyrrolidone (NMP) were purchased from Nacalai tesque INC. (Kyoto, Japan). Ethyl acetate was purchased from Godo CO., LTD. (Tokyo, Japan). n-Hexane, deuterium oxide (D_2O), sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 (TMS), dimethyl sulfoxide-d_6, containing 0.05 wt% of TMS (volume fraction) for ^{1}H-NMR measurement, triethylamine, dihydrogenphosphate dihydrate, disodium hydrogenphosphate 12-H_2O, sodium chloride (NaCl), hydrochloric acid (HCl), acetic acid, oxaliplatin, phosphate-buffered saline (PBS), and penicillin-streptomycin solution (x100) were purchased from Wako pure chemical corporation (Osaka, Japan). Acetic anhydride, 1,5-diaminopentane (DAP), and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC/HCl) were purchased from Tokyo chemical industry CO., LTD. (Tokyo, Japan). Singlet oxygen sensor green (SOSG) and Sulfo-Cy5, was purchased from Thermo Fisher Scientific K.K. (Tokyo, Japan). Endoperoxide (EP) reagent was purchased from Wakenbtech CO., LTD. (Kyoto, Japan). Methoxy-poly(ethylene glycol)-amine (MeO-PEG-NH_2; molecular weight (MW): 12k) was purchased from NOF Corp. (Tokyo, Japan). Dialysis membrane (molecular weight cut-off (MWCO) = 6,000-8,000) was purchased from Spectrum Laboratories, INC. (CA, US). Vivaspin 20-50k were purchased from GE Healthcare Science (Tokyo, Japan). Phosphate buffer (PB), DMEM were purchased from Sigma Aldrich Japan (Tokyo, Japan). EZ SPHERE 96 well plate was purchased from AGC TECHNO GLASS Co., Ltd. (Shizuoka, Japan). Human

pancreatic cancer BxPC3 cells were obtained from the American Type Culture Collection (Manassas, VA).,

2. Equipment and measurements

The synthesized products were evaluated by nuclear magnetic resonance spectrometer (NMR 400 MHz : ECS 400, Japan Electron Optics Laboratory, Ltd., Tokyo, Japan), Gel Permeation Chromatography (GPC; TOSOH HLC- 8220 GPC, column; SuperAW 3000×2, superAW 4000, TSK gel column, superAW-L, carrier; DMF +10mM LiCl, flow speed; 0.3 mL/min, temperature; 40 ^{o}C, Tosoh Corp., Tokyo, Japan), and High Performance Liquid Chromatography (HPLC; Jasco RI-930, UV-1575, column; Tricorn Superdex200 (GE Healthcare Bio-Science KK, Jasco corp., Tokyo, Japan), carrier; 10mM PB 150mM NaCl, pH7.4, flow speed; 0.75mL/min, temperature; room temperature). The size and PDI of the micelles were evaluated by dynamic light scattering (DLS; Zeta sizer Nano ZS, Spectris Co., Ltd., Tokyo, Japan). The fluorescence of the samples was evaluated by fluorescence spectroscopy with Nano Drop (ND-3300, Spectrofluorometer 2.6.0, Coleman Technologies, PA, US.) and by Microplate Reader (Spark 20M, TECAN). The micelles were purified by centrifuge (Universal cooling centrifuge 5922, KUBOTA Corp., Tokyo, Japan.).

3. Synthesis of Poly(ethylene glycol)-Poly(Aspartic acid)

Synthesis of PEG-Poly(Aspartic acid) (PEG-P(Asp)) was done by NCA polymerization and deprotection with NaOH [1]. MeO-PEG-NH_2 was dissolved in dichloromethane with an argon atmosphere. BLA-NCA was dissolved in *N*,*N*-dimethylformamide and dichloromethane. These solutions were mixed and stirred for 3 days at 35 degrees. Then, the mixture was added dropwise

into mixture of ethyl acetate/ hexane (3:2, v/v) for precipitating the polymers. PEG-poly(benzyl-L-aspartate) (PEG-PBLA) was obtained as a white powder by filtration and vacuum dry. PEG-PBLA was dissolved into 0.25 N of NaOH aqueous solution (5 molar equivalent to benzyl group), then stirred for 1 h at room temperature to remove the protecting group. The solution was purified by dialysis against milliQ water 5 times (MWCO: 6,000-8,000 Da). PEG-P(Asp) with 75 units of aspartic acid was obtained as white powder by lyophilization.

4. Synthesis of PEG-P(Asp)-Cy5

The ω-terminal end of PEG-PBLA was labelled with sulfo-Cy5-NHS ester by stirring in DMSO (50 mg/mL) at room temperature for 24 h. The reaction solution was precipitated against mixture of ethyl acetate/ hexane (3:2, v/v), followed by filtration under reduced pressure. The benzyl group of obtained PEG-PBLA-Cy5 was deprotected according to the former Section 3 to obtain PEG-P(Asp)-Cy5.

5. Synthesis of PEG-poly([5-aminopentyl]-α,β-aspartamide)

The synthesis of PEG-poly([5-aminopentyl]-α,β-aspartamide) (PEG-P(Asp-AP)) was done by post-polymerization aminolysis using 1,5-diaminopentane [1]. PEG-PBLA was dissolved in dehydrated *N*-methyl-2-pyrolidone. After 12 h stirring, mixture solution of distilled 1,5-diaminopentane (100 molar equivalent to BLA unit) and dehydrated NMP was slowly dropped (1drop/sec) in reaction solution with ice bath. After 1 h stirring, excess amount of 5N HCl was dropped in the reaction solution for neutralization. The resulting solution was dialyzed (MWCO: 6,000-8,000 Da) against 0.01 N of HCl aqueous solution once and milliQ water 5 times. PEG-P(Asp-AP) with 75 Asp-AP units was obtained as white powder by lyophilization.

6. Preparation of Cy5-PIC/m

PEG-P(Asp)-Cy5 and PEG-P(Asp-AP) were dissolved in 10 mM phosphate buffer (PB, pH 7.4, 0 mM NaCl) separately to prepare a 1 mg/mL polymer solution. Then, the polymers were mixed at charge (carboxylate to amine) equivalent concentration. After 2 minutes of agitation, 1 mg of EDC/HCl was added to the reaction solution. After 24 h incubation, the Cy5-PIC/m were purified by ultrafiltration 5 times (MWCO: 50,000 Da).

7. Conjugation of SOSG to Cy5-PIC/m

EDC/HCl (1 mg) and SOSG (0.1 mg) were mixed into 100 mL of Cy5-PIC/m solution (1 mg/mL), and stirred overnight at room temperature. The SOSG@Cy5-PIC/m were obtained by ultrafiltration 5 times (MWCO: 50,000 Da).

8. In vitro $^1O_2$ *detection of dual-labelled micelles*

Twenty microliters of SOSG@Cy5-PIC/m solution (200 μg/mL) were added to each well of a 96 well plate. Then, 20 μL of different concentrations (0 - 4 mM) of endoperoxide (EP) were mixed in SOSG@Cy5-PIC/m solution. Immediately after heating up to 40 ^oC, the fluorescent curves were measured with 460 nm excitation to evaluate the ability of SOSG@Cy5-PIC/m to measure $^1O_2$.

9. Cellular uptake assay

BxPC3 cells were seeded in 96-well plate (monolayer culture) and EZ SPHERE 96-well plate (Spheroid) with the concentration of 5 x 10^4 cells/well in DMEM containing 10 vol% FBS

and 1% penicillin and streptomycin. After 24 h for monolayer culture and 72 h for spheroid culture, the supernatant was gently removed and the systems were softly rinsed twice using PBS. After addition of 180 μL of cell culture media, 20 μL of SOSG and SOSG@Cy5-PIC/m solutions were added to each well. Cellular uptake of SOSG@Cy5-PIC/m were quantitatively imaged by confocal laser scanning microscopy (CLSM; Zeiss 780 LSM) at different incubation time (1, 3, 6, 24 h).

10. Detection of in situ $^1O_2$ generation by BxPC3 cell line

BxPC3 cells were seeded in EZ SPHERE 96-well plate with the concentration of 5 x 10^4 cells/well by using 10 vol% FBS containing DMEM with penicillin and streptomycin. After 72 h of preincubation, the supernatant was gently removed and the systems were softly rinsed with PBS twice. After addition of 180 μL of cell culture media, 20 μL of SOSG and SOSG@Cy5-PIC/m solutions were added to each well. After 24 h incubation, supernatant was gently removed and the systems were rinsed with PBS twice. After addition of 180 μL of 10% FBS containing DMEM with penicillin and streptomycin, 20 μL of oxaliplatin solution (1 μM) or saline was added to each well, following $^1O_2$ generation was quantitatively imaged by using CLSM at 1-, 3-, 6- and 24-h.

References

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