Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B

Electronic Supplementary Information (ESI)

Extracellular vesicle containing fullerene derivatives prepared by an exchanging reaction for photodynamic therapy

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Materials and Methods

Materials

C_{60}-**1** and C_{60}-**2** were synthesized as described in previously published papers.[1,2] γ-CDx was purchased from Fuji Film Wako Pure Chemical Industries Ltd (Tokyo, Japan). 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) was purchased from Funakoshi Co., Ltd (Tokyo, Japan). 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI) was obtained from Molecular Probes, Inc. (Eugene, OR, USA). Photofrin and 9,10-anthracenediylbis(methylene)dimalonic acid (ABDA) were purchased from Takeda Pharmaceutical Co., Ltd (Osaka, Japan) and Merck (Milwaukee, WI, USA), respectively. Cell lines, human cervical adenocarcinoma cell (HeLa), murine colon carcinoma cell (Colon26) and human lung cancer cell (A549), were maintained in Minimum Essential Medium (MEM, Thermo Fischer Science) containing 10 % fatal bovine serum (FBS, Thermo Fischer Science) and 1 % antibiotics or Dulbecco's Modified Eagle Medium (DMEM, Thermo Fischer Science) containing 10 % FBS, 1 % antibiotics, and 1 % glutamic acid.

Isolation and purification of EVs from lemon juice

Lemons were purchased from a local supermarket. Lemons were peeled and then squeezed to obtain the juice. The juice was sequentially centrifuged at 1,000 g for 10 min, 2,000 g for 20 min, 4,000 g for 30 min, and 8,000 g for 60 min to remove large particles and precipitations. The supernatant was passed through a 0.2 μm filter and then centrifuged at 36,000 g for 2 h. After removal of the supernatant, the pellet was resuspended in 5 mL milliQ. All centrifugations were performed at 4 °C. The concentration of protein and lipid in the sample was determined using BCA protein assay and phospholipid assay, respectively. Their size distribution was evaluated by nanoparticle tracking analysis (NTA). Representative morphology was observed by transmission electron microscopy with negative staining using 2.0 % phosphotungstic acid (TEM).

Preparation of liposomes

An appropriate amount of DMPC was dissolved in chloroform. The solvent of the solution thus prepared was removed by evaporation under a gentle stream of nitrogen, followed by a period whereby the solution was kept under vacuum to remove any solvent traces. The thin lipid films thus obtained were hydrated on the wall of the vial with an appropriate amount of water at ~45°C. The hydrated materials were subjected to five consecutive freeze–thaw cycles (at −195°C and 50°C) to produce unilamellar vesicles, which were extruded 11 times through 0.1 μm pores using a LiposoFast miniextruder sourced from Avestin at a temperature above that of the phase transition. The resulting liposomes were uniform in size and had a

diameter of approximately 150 nm.

Preparation of γ-CDx•C_{60}-1 and γ-CDx•C_{60}-2 complexes

The aqueous solutions of γ-CDx•C_{60}-**1** and γ-CDx•C_{60}-**2** complexes were prepared as described in previous studies. Briefly, γ-CDx and fullerenes were mixed (γ-CDx, 12 μmol; fullerenes, 3 μmol) in solid state and high-speed vibration milling (MM 200; Retsch Co., Ltd, Haan, Germany) was achieved to form the complex (25 Hz, 30 min). The resulting complex was extracted with MilliQ and the precipitation was removed by centrifugation (14000g, 20 min). The formulation was confirmed by NMR and UV-Vis spectroscopy. The aqueous solutions were diluted to a final concentration of 0.20 mM.

Preparation of LipC_{60}-1, LipC_{60}-2, EVsC_{60}-1, and EVsC_{60}-2

LipC_{60}-**1** and LipC_{60}-**2** were prepared via an exchange reaction between liposome and γ-CDx•C_{60}-**1** or γ-CDx•C_{60}-**2** complexes, as described previously. EVsC_{60}-**1** and EVsC_{60}-**2** were prepared *via* an exchange reaction between EVs and γ-CDx•C_{60}-**1** or γ-CDx•C_{60}-**2** complexes by maintaining at 40 ^{o}C for 7 days. The final concentrations of the relevant components were [C_{60}-**1** or C_{60}-**2**] = 0.10 mM, [lipids] = 1.00 mM ([C_{60}-1 or C_{60}-2]/[lipids] = 10 mol%) and [EVs] = 0.25 mg•mL^{-1}). Proceeding of exchanging reactions were monitored by UV-Vis spectroscopy. The absorbance spectra of Lip/C_{60}-**1**, Lip/C_{60}-**2**, EVs/C_{60}-**1**, and EVs/C_{60}-B prepared in the current study were recorded using a UV-3600 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). In addition, changes in representative peaks from fullerenes were tracked by NMR. The size distribution of the EVs loading fullerenes were addressed by NTA. The samples were observed by TEM with negative staining.

Detection of photo-triggered conversion of reactive oxygen species

The reactive oxygen species generated as a consequence of the implementation of the ABDA bleaching method (Sigma-Aldrich Corp.) were detected through a previously reported method. ABDA was used as a dimethyl sulfoxide (DMSO) solution ([ABDA] = 2.50 mM). The final concentrations of the fullerene derivatives and ABDA in the mixture were 20 and 12.5 μM. Oxygen gas was bubbled through all of the sample solutions for 30 min before photoirradiation to ensure aerobic conditions. Photoirradiation was performed using a xenon lamp (SX-UID500X, 500 W; Ushio Inc., Tokyo, Japan) equipped with a long-pass filter with a cut-off at 620 nm. The light was cooled by making it pass through a water filter. The power of the light was 16 mW cm^{-2} (over 620 nm) at sample level.

Photodynamic activity experiments

HeLa cells, Colon26 cells, and A549 cells were maintained in CO_2-Independent Medium (Gibco BRL) supplemented with 10% feral calf serum at 37 °C in 5% CO_2. In the experiments conducted to determine the photodynamic activities of PF, Lip/C_{60}-**1**, Lip/C_{60}-**2**, EVs/C_{60}-**1**, and EVs/C_{60}-**2**, the cells were seeded into 48-well culture plates at a density of 8.55×10^4 cells per well. After being allowed to grow overnight, the cells were incubated with PF, LipC_{60}-**1**, Lip/C_{60}-**2**, EVs/C_{60}-**1**, and EVs/C_{60}-**2** for 24 h in the dark. The cells were then washed with phosphate-buffered saline (PBS) and exposed to light for 30 min at room temperature. Light irradiation was conducted using a xenon lamp (MAX-301, 300 W; Asahi Spectra Co., Ltd, Tokyo, Japan) equipped with a VIS mirror module (385–740 nm) and a long-pass filter with a cut-off at 610 nm. The power of the light was 9 mW cm^{-2} (610–740 nm) at cell level. The viability of the cells was measured as the ratio (%) of the number of viable cells in the treatment groups to the number of viable cells in the untreated group. A WST-8 assay was carried out 24 h after photoirradiation using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions.

Preparation of HP-β-CDx•NBD-Cl complexes

NBD-Cl was used as a model compound instead of C_{60} derivatives to measure intracellular uptakes. NBD-Cl (5.0×10^{-6} mol) and 2-Hydroxypropyl-β-cyclodextrin (HP-β-CDx, 1.0×10^{-5} mol) were placed in an agate capsule with two agate-mixing balls. The materials were then mixed vigorously at 25 Hz for 30 min using a high-speed vibration mill. The resulting solid mixture was suspended in H_2O (1.5 mL) to produce a yellowish suspension, which was centrifuged for 20 min (4,500 rpm, 25 °C), allowing for the removal of the non-dispersed NBD-Cl from the solution. The concentrations of NBD-Cl in the HP-β-CDx complex were determined based on the absorbance characteristics of the corresponding solutions at 343.5 nm (the molar absorption coefficients for the water-soluble HP-β-CDx•NBD were $\varepsilon_{343.5} = 1.37 \times 10^4$ dm^3 mol^{-1} cm^{-1}). This value was found to be 0.88 mM in aqueous solution. this aqueous solution was diluted to a final concentration of 0.2 mM.

Preparation of Lip/NBD and EVs/NBD

Lip/NBD and EVs/NBD were prepated via an exchange reaction between exosome and HP-β-CDx•NBD-Cl complexes by heating at 80 °C and 40 °C, respectively, for 1 and 7 days. The final concentrations of the relevant components were [NBD-Cl] = 0.10 mM, [lipids] = 1.00 mM ([NBD-Cl]/[lipids] = 10 mol%) and [EVs] = 0.25 mg•mL^{-1}).

Preparation of Lip/NBD-DiI and EVs/NBD-DiI

DiI/DMSO was added to Lip/NBD and EVs/NBD, stirred for 20 min, and centrifuged at 120,000 g for 70 min. The centrifugation was performed at 4 °C. The supernatant was removed and resuspended in milliQ.

Intracellular uptakes of Lip/NBD and EVs/NBD

HeLa cells, Colon26 cells, and A549 cells were seeded into 12-well culture plates at a density of 1.0 × 10^5 cells per well (1.0 mL). After allowing the cells to grow for 24 h, they were incubated in a medium that included an added aqueous solution of solution of Lip/NBD and EVs/NBD for 24 h in the dark at 37 ^oC (final NBD-Cl concentration: [NBD-Cl] = 10 μM). The cells were then washed with PBS and detached using trypsin containing 0.5%–EDTA (5.3 mM, 100 μL). After performing the detachment treatment for 5 min and quenching trypsin via the addition of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (500 μL), the cells were collected by implementing a centrifugation step at 1,500 g that lasted for 5 min. The cells were then washed with PBS (500 μL) and disrupted using a vortex mixer after the addition of RIPA buffer (Nakalai tesque Inc.) (500 μL). After centrifugation (1,500 g for 5 min), NBD-Cl was extracted with ethyl acetate (700 μL). After another centrifugation step (1,500 g for 5 min), the fluorescence intensity was recorded (excitation wavelength, λex: 460 nm). EVs and liposomes were disrupted by adding Triton X-100 to the same amount of EVs/NBDs and Lip/NBDs added to cells, and NBD-Cl was extracted by mixing with ethyl acetate (700 μL). The fluorescence intensity of the extracted dye was used as 100% to compare cellular uptake. The same cell experiments described above were also performed using Lip/NBD-DiI and EVs/NBD-DiI, and cell uptake was confirmed using confocal lasar microsopy (LSM700, Carl Zeiss, Germany).

Photodynamic activity *in vivo*

Tumor xenograft mice were established by transplantation of Colon26 (1.0 ×10^5 cells) to the right femur of Balb/c (male, 4-week-old, 18 g). After 7 days incubation, the mice were treated with PBS, Lip/C_{60}, or EVs/C_{60} (C_{60}, 0.1 mM) via *i. v.*. After 24 h-post injection, the photo-irradiation was carried out to the mice for 1 h (>600 nm, 15 mW). Afterward, the tumor volume and body weight were measured at each time point. The tumor volume was calculated by following equation.

$$V = (\text{Long axis}) \times (\text{Short axis})^2/2$$

All animal experiments were performed in accordance with the Guidelines for care and use of laboratory animal of Osaka Metropolitan University and were approved by the ethics committee for animal welfare of Osaka Metropolitan University (accreditation, S0101).

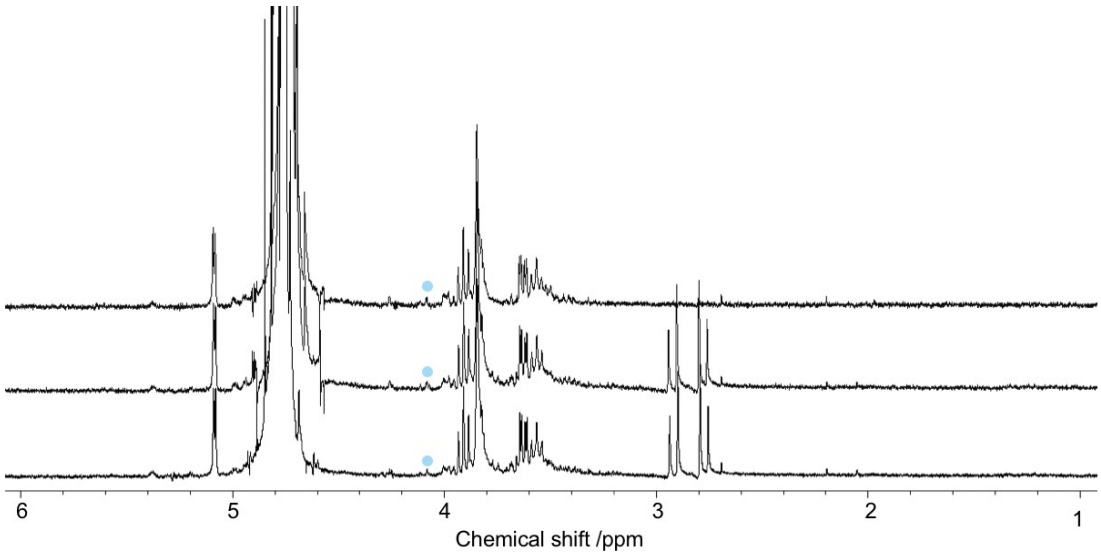


Fig. S1 Proceeding of introduction of C_{60} derivatives into membrane of EVs via exchanging reaction. ^{1}H-NMR spectra of C_{60}-**1** complexed with γ-CDx in D_2O (top panel). ^{1}H-NMR spectra of the mixture of C_{60}-1 complexed with γ-CDx and EVs in D_2O at 3 days (middle panel). ^{1}H-NMR spectra of the mixture of C_{60}-**1** complexed with γ-CDx and EVs in D_2O at 7 days.

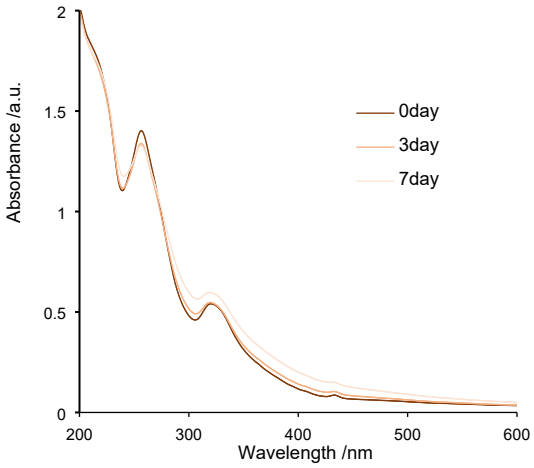


Fig. S2 Changes in UV-Vis absorption spectra of C_{60}-**1** *via* exchanging reaction between γ-CDx and EVs.

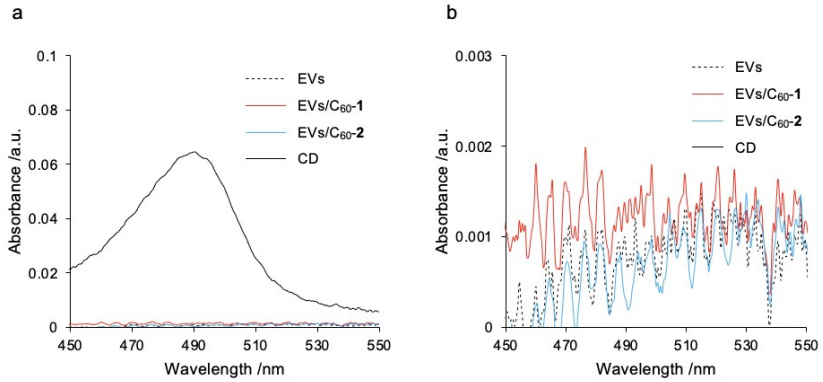


Fig. S3 Sugar determination by phenol-sulfonic acid method in dispersion of EVs and EVs/C_{60} derivatives. (a) UV-Vis spectra of EVs (dashed black line), EVs/C_{60}-**1** (solid red line), EVs/C_{60}-**2** (solid blue line), and CD (solid black line) were treated with phenol and sulfonic acid. EVs samples were isolated by ultracentrifugation to remove free CD. (b) Enlarged graph for (a).

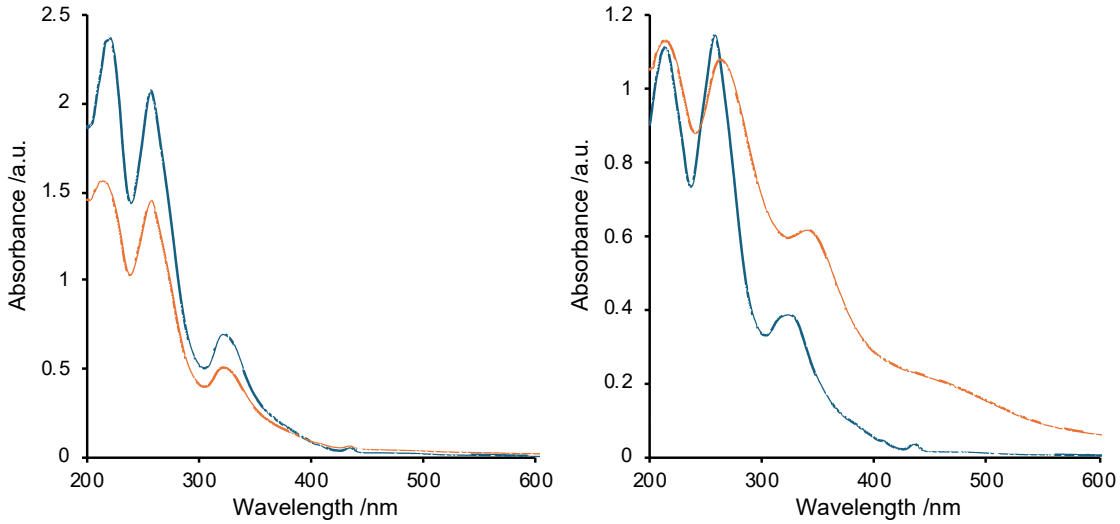


Fig. S4 Changes in UV-Vis absorption spectra of C_{60} derivatives *via* exchanging reaction between γ-CDx and liposome. (a) C_{60}-**1**·γ-CDx was co-incubated with DMPC liposome and the UV-Vis absorption spectra was measured at 0 (blue) and 2 h (red). (b) C_{60}-**2**·γ-CDx was co-incubated with DMPC liposome and the UV-Vis absorption spectra was measured at 0 (blue) and 2 h (red).

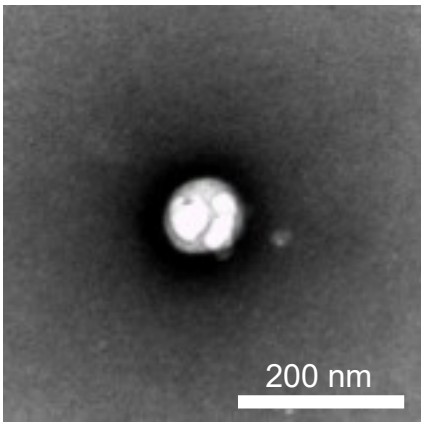


Fig. S5 Representative morphological image of EVs/C_{60}-**1**. The samples casted on the grid were stained with 0.1 % phosphotungstic acid. The samples were observed by TEM (acceleration voltage, 80 kV).

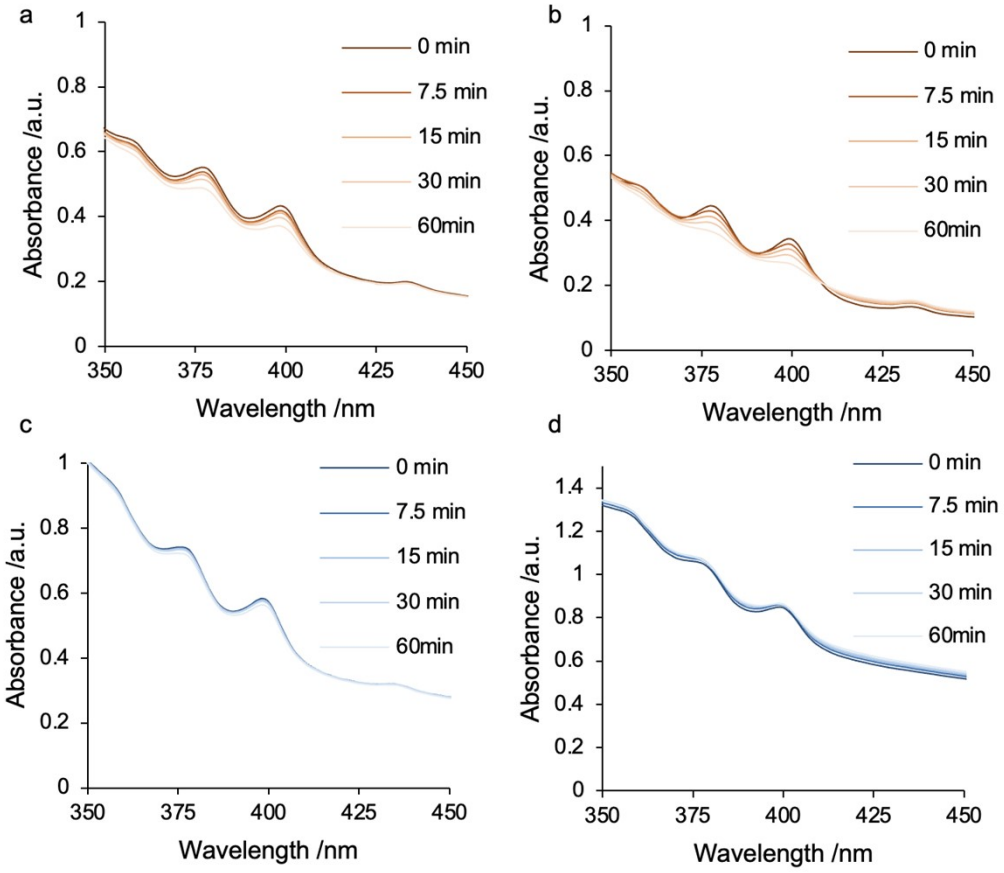


Fig. S6 Consumption of ABDA *via* photo-triggered oxidation with singlet oxygen generated by (a) EVs/C_{60}-**1**, (b) Lip/C_{60}-**1**, (c) EVs/C_{60}-**2**, and (d) Lip/C_{60}-**2**.

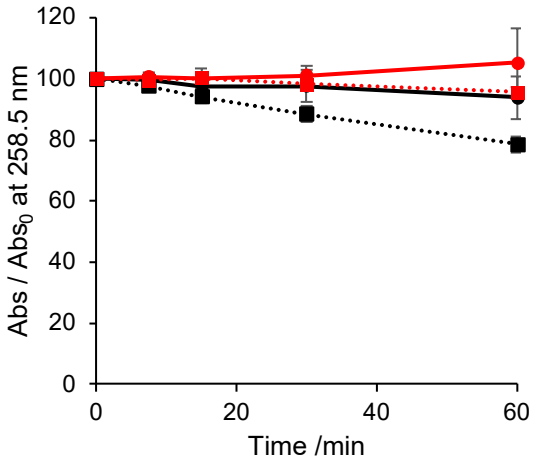


Fig. S7 Stability of fullerenes against photo-irradiation. EVs/C_{60}-**1** (black, circle), EVs/C_{60}-**2** (red, circle), Lip/C_{60}-**1** (black, square), and Lip/C_{60}-**2** (red, square) were irradiated and changes in absorption of fullerene at 258.5 nm were plotted.

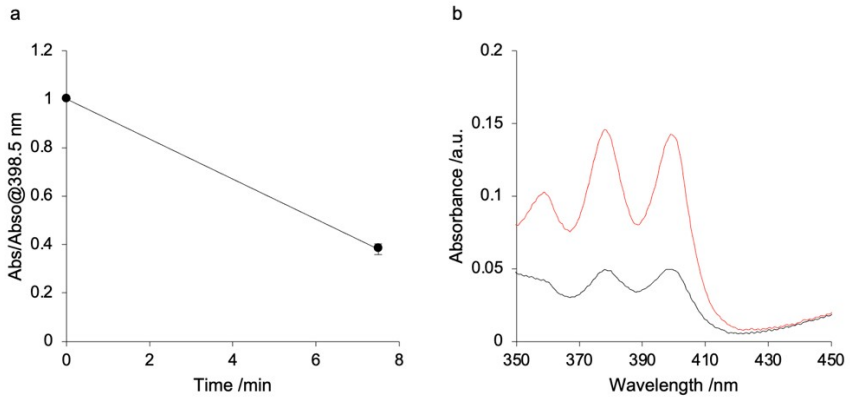


Fig. S8 Photodynamic activity of methylene blue. (a) Conversion of ABDA to endoperoxide via oxidation by $^1O_2$. (i) Detection of the generated $^1O_2$ using ABDA. Methylene blue (20 μM) were co-incubated with ABDA (12.5 μM) and the resulting dispersion were irradiated at 680-800 nm. The consumption of ABDA were quantified with changes in absorbance at 398.5 nm. Data represents mean ± SD (n = 3). (b) Spectral changes in ABDA during the photoirradiation to methylene blue solution. (Red, 0 min; black, 7.5 min)

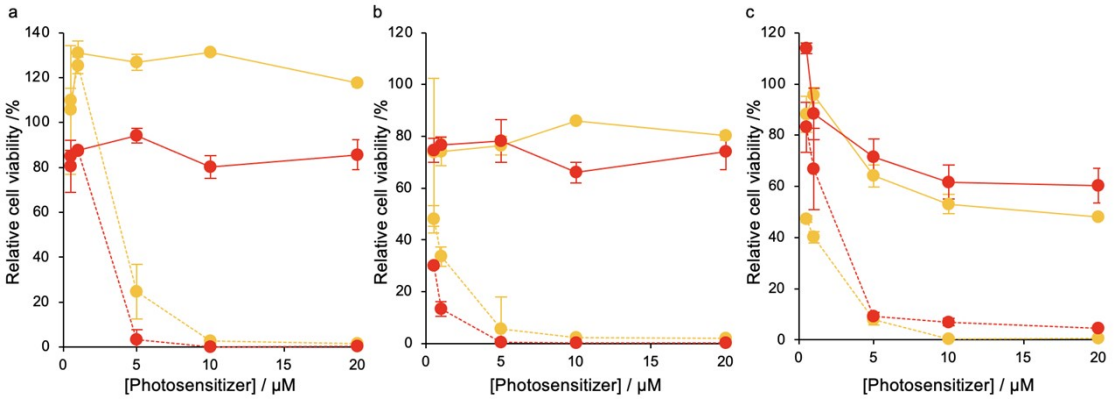


Fig. S9 Photodynamic activity of EVs/C_{60}-**1** toward cancer cells. Colon26 (a), HeLa (b), or A549 (c) were exposed to EVs/C_{60}-**1** (red), or Lip/C_{60}-**1** (yellow). The treated cells were irradiated with optimal wavelength (dashed line) and the other group was incubated under dark condition (solid line). After additional 24 h, the cell viability was estimated by WST-8. The data represents mean ± SD (n = 3).

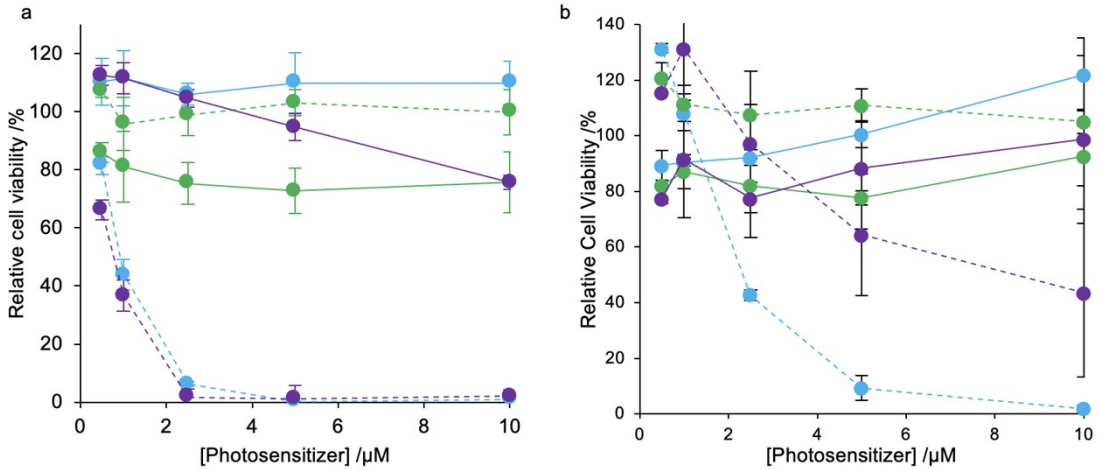


Fig. S10 Photodynamic activity of EVs/C_{60}-**2** toward cancer cells. HeLa (a), or A549 (b) were exposed to EVs/C_{60}-**2** (blue), Lip/C_{60}-**1** (green), or Photofrin (purple). The treated cells were irradiated with optimal wavelength (dashed line) and the other group was incubated under dark condition (solid line). After additional 24 h, the cell viability was estimated by WST-8. The data represents mean ± SD (n = 3).

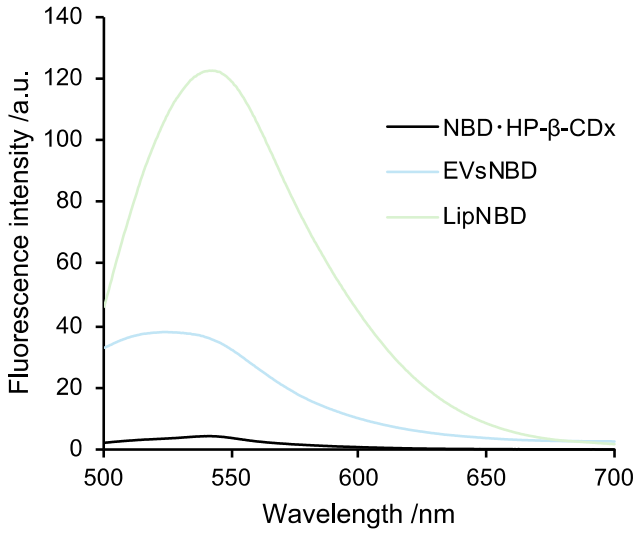
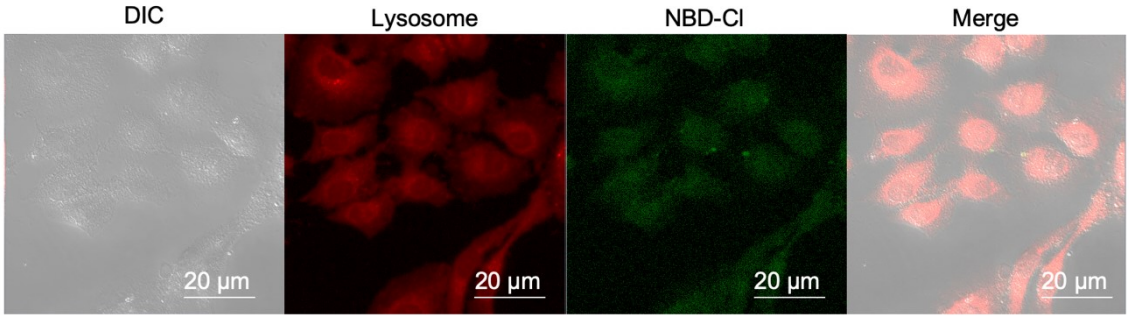


Fig. S11 Changes in fluorescence of NBD *via* exchanging reaction.

Fig. S12 Subcellular distribution of delivered NBD-Cl/EVs. Subcellular distribution of delivered EVs and cargo molecules. Colon26 cells were exposed to NBD-Cl/EVs for 24 h. After washing with PBS, the sample was observed *via* confocal laser scanning microscopy. Lysosome was stained with Lysotracker Red.



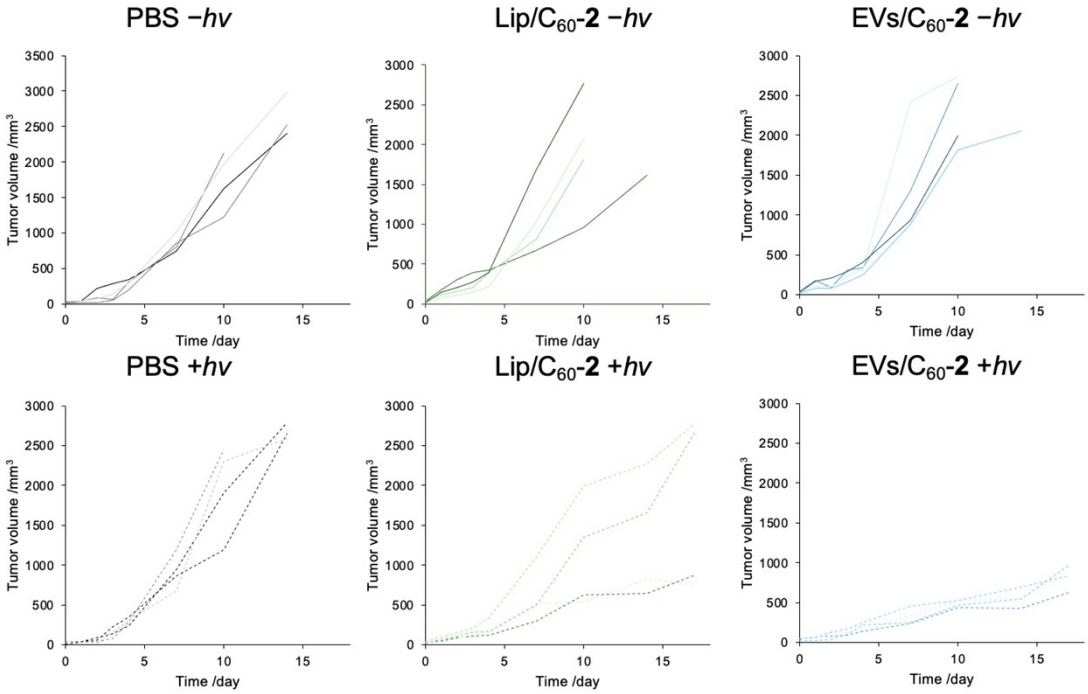


Fig. S13 Tumor growth curve in individual mice. Tumor xenograft mice which were established by transplantation of Colon 26 cells were treated with PBS (black), Lip/C_{60}-**2** (green), or EVs/ C_{60}-**2** (blue). After 24 h post-injection, the tumor was irradiated with or without light for 1 h. The tumor volume was measured at each time point.

References for Supporting Information

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