Electronic Supplementary Information (ESI)

Enhancement of the photocytotoxic efficiency of sub-12-nm therapeutic polymeric micelles with increased co-localisation in mitochondria

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Experimental Section

1. Materials

1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy poly(ethylene glycol)] (DSPE-mPEG; Avanti Polar Lipids, Alabaster, AL, USA), methoxy poly(ethylene glycol) (mPEG; Sigma-Aldrich, St. Louis, MO, USA), hypericin (HY; Sigma-Aldrich), chloroform (Amresco, Solon, OH, USA), phosphate-buffered saline (PBS; Sigma-Aldrich), dimethyl sulphoxide (DMSO; Sigma-Aldrich) and ultrapure water (DW; Welgene Inc., Daegu, Republic of Korea) were all used as-received.

2. Preparation of the drug delivery systems (DDSs)

Three kinds of drug delivery systems (DDSs) were used in combination with HY: an aggregated form (HY-agg), a PEGylated form (HY-mPEG) and our engineered polymeric micelle (HY-M). Solutions of HY-agg and HY-mPEG were prepared as previously reported [s1] except for the length of the mPEG: mPEG with an average molecular weight of 2000 was used instead of PEG400. HY-agg was prepared by dissolving a solution of HY in DMSO (16 mg/mL). HY-mPEG was prepared by mixing equal volumes of DMSO solutions having the same molar concentrations of HY and $mPEG_{2000}$ (62.5 μL, 31.72 mM each). For the synthesis of HY-M, three different kinds of polymeric lipids were used that differed in the molecular weight of the mPEG (350, 2000 and 5000). A polymeric lipid film with HY was first prepared by mixing HY (200 μL, 0.2 mg/mL) and DSPE-mPEG (10 mg/mL) in chloroform. The added volume of DSPE-mPEG solution was determined by the molar ratio (DSPE-mPEG:HY = 10:1 for each of $DSPE\text{-}mPEG_{350}$, $DSPE\text{-}mPEG_{2000}$ and $DSPE\text{-}mPEG_{5000}$, and 1:1 and 4:1 for $DSPE\text{-}mPEG_{2000}$) with the same concentration as above. After complete evaporation of the chloroform, the dried lipid film was hydrated with 1 mL water at 75°C in an ultrasonic bath for 5 min to obtain an optically clear suspension. To eliminate oversized particles or aggregates, the solution was filtered through a 0.1-μm membrane (Millipore, Billerica, MA, USA). Then, to eliminate unincorporated HY or undersized nanoparticles, triple rinsing on a 30 000 MWCO centrifuge filter (Millipore) with DW was performed. A clear solution of HY-M was finally obtained.

3. Characterisation of the polymeric micelles

3.1 Spectral absorbance and fluorescence measurements

The spectral absorbance and fluorescence of HY dissolved in 100% DMSO were measured with a SpectraMax Plus 384 absorbance microplate reader and Gemini XPS fluorescence microplate reader (Molecular Devices, Sunnyvale, CA, USA). The absorbance within 400–800 nm and fluorescence emissions within 510–800 nm were measured for 490-nm-wavelength excitation (Figure S1A). The spectral absorbance of HY-M in DW was determined; 5 nm redshifting of the peak absorbance was observed (Figure S1B). The previously reported method was used to determine the HY concentration inside the nanoparticles and the HY-DDS-treated cells.[2–4] For that reason, the spectral measurement and the dose-dependent absorbance (at 595 nm wavelength) analysis of HY were performed with different lysis solutions: DMSO:lysis buffer (M-PER mammalian protein extraction reagent; Thermo Fisher Scientific, Lake Barrington, IL, USA) = 1:1 for the evaluation of the DDS-dependent cellular uptake of HY, and DMSO:lysis buffer:DW = 1:1:2 for the determination of the incorporation efficiency and the drug loading of the micelles (Figure S1C).

3.2 Dynamic light scattering measurements

The hydrodynamic size, polydispersity index (PI) and zeta potential of the micelles were measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS90 (Malvern Instrument Ltd., Worcestershire, UK). The suspensions were diluted 20-fold in DW and measured (Figure S2A, Table S1). Once DSPE-mPEG$_{2000}$ had been selected as the most suitable carrier for HY, the size was remeasured in PBS solution (Figure 2A).

3.3 Incorporation efficiency, drug loading and cost efficiency

The incorporation efficiency and drug loading were calculated using the absorbance results (Figure S1, ESI Section 3.1, and Table S1) with Equations 1 and 2, which are similar to those previously reported.[s2,s5] The total cost required to engineer HY-M to encapsulate 1 μM of HY is referred to as the cost efficiency and was calculated using Equation 3. The price used was the commercial price listed on the online web pages of each material. Because DSPE-mPEG$_{2000}$ gave the smallest-sized micelles (Figure S2A), more specific evaluations with

DSPE-mPEG$_{2000}$ were performed by altering the molar ratio between the polymeric lipid and the agent (Figure S2B). Linear regression analysis was performed to generate the dose-dependent equations for the HY concentration

$$\text{Incorporation efficiency (\%)} = \frac{\text{mass of HY incorporated in micelles (g)}}{\text{mass of HY introduced (g)}} \times 100 \quad (1)$$

$$\text{Drug loading (\%wt/wt)} = \frac{\text{mass of HY incorporated in micelles (g)}}{\text{mass of polymer introduced (g)}} \times 100 \quad (2)$$

$$\text{Cost efficiency (\$/µmole HY)} = \frac{(\text{cost of polymer introduced} + \text{cost of 1 µmole of HY})}{\text{incoroporation efficiency}} \times 100 \quad (3)$$

3.4 Morphological and size studies of micelles by transmission electron microscopy

Transmission electron microscopy (TEM; JEM-2100F, JEOL Ltd., Tokyo, Japan) at 200 kV was used to study the morphology and size of the micelles. Samples were prepared on an ultrathin carbon film on a holey/lacey carbon support film, which was supported on a 400-mesh copper grid (Ted Pella Inc., Redding, CA, USA). After allowing a drop of a suspension to spread on the grid for 3–5 min, the excess solution was removed with a filter paper until the surface of the grid was nearly dry. Then, the sample was prepared using two different staining methods: negative staining using a 1% solution of sodium phosphotungstate (PTA; Sigma-Aldrich) in DW, for which the pH was adjusted to 7.2–7.4 with 0.1 N NaOH, and non-staining. The negative staining was done by placing a drop of the PTA solution on the prepared grid and removing the excess solution with a filter paper after 1 min. After the staining or non-staining process, the grid was dried for about 15–20 min in a covered Petri dish, then examined by TEM (Figures 2B and S3).

4. Cell culture

Malignant brain tumour cells (human glioblastoma U251MG cells) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and grown in Dulbecco's modified Eagle's medium consisting of Nutrient Mixture F-12 (DMEM/F12). The medium was supplemented with 10% foetal bovine serum, antibiotics [penicillin (100 I.U./mL) and streptomycin (100 µg/mL)] and 0.1 mM of MEM nonessential amino acids solution. All

solutions were purchased and used as received from Gibco (Grand Island, NY, USA). Cells were maintained and incubated at 37°C in an atmosphere of 5% CO_2.

5. Determination of cytotoxicity and cellular uptake

5.1 Cytotoxicity

To determine the dark-cytotoxicity of HY and HY-containing nanoparticles, 5×10^4 U251MG cells were seeded in a 24-well cell culture plate (TPP Techno Plastic Products AG, Trasadingen, Switzerland) with 1 mL of complete DMEM/F12 medium in quadruplicate for each sample. After 24 h of incubation, the medium was replaced with an HY-DDS (HY-agg, HY-mPEG and HY-M)-containing media having different concentrations of HY (1, 2.5, 5 and 10 μM for HY-agg, and 2.5, 5 and 10 μM for HY-mPEG and HY-M). The volume of added HY-DDS did not exceed 1% of the total volume of the applied medium. After 2 h of incubation, the wells were washed with PBS and replaced with drug-free medium and incubated an additional 24 h. To assess cell viability/cytotoxicity, WST-1 reagent (Daeillab Service Ltd., Seoul, Republic of Korea) was added to each well at 10 vol% of the medium. After 1 h of incubation, the absorbances of the supernatants were measured using the absorbance microplate reader at a wavelength of 450 nm. The normalised absorbance is called the viability (Figure S4A, Figure 3A). All procedures involving the HY-DDSs were performed under light-restricted conditions.

5.2 Cellular uptake

Next, 1×10^5 cells of U251MG cells were seeded in a 12-well cell culture plate (TPP Techno Plastic Products AG) with 2 mL of complete DMEM/F12 medium in quadruplicate for each sample and incubated for 24 h. The medium was then replaced with HY-DDS-containing complete media having different concentrations of HY (1, 2.5, 5 and 10 μM for each DDS) and incubated for different times (15 and 30 min, 1, 2, 3, 4, 6, 8, 12 and 24 h). After incubation, the cells were collected with 60 μL of lysis buffer per well, and an equal volume of DMSO was added to each sample. After vortexing, the cells were centrifuged at 13,000 rpm for 20 min and the HY and protein concentrations were measured in the supernatants. The HY concentration was calculated as described above (ESI Section 3.1) and the protein

concentration was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies/Thermo Scientific, Wilmington, DE, USA). All procedures involving the treatment of the HY-DDSs were performed on ice or at 4°C and under light-restricted conditions. The cellular uptake of HY in the U251MG cells was calculated using Equation 4 and used to determine the time- and dose-dependent cellular uptake of HY (Figure S4B). The data was fitted to a nonlinear one phase association regression model (Equation 5). Parameters computed from the model are listed in Table S2. The cellular uptake evaluation method was used to calculate the photocytotoxic efficiency.

$$\text{Cellular uptake of HY (A.U.mL/mg)} = \frac{\text{measured absorbance of supernantant (A.U.)}}{\text{measured protein concentraion of supernant (mg/mL)}} \quad (4)$$

$$Y = Y_0 + (\text{Plateau} - Y_0) \times (1 - e^{(-K\,X)}) \quad (5)$$

Y_0 = Y value when X (time) is zero, Plateau = Y value at infinite times, K = rate constant

6. Evaluation of the photocytotoxic efficiency and intracellular co-localisation of HY in HY-agg, HY-mPEG and HY-M

6.1 Light irradiation system (LIS) and dosimetry

To evaluate the effectiveness of the therapeutic polymeric micelles, which is expressed as the photocytotoxic efficiency, an LIS was used to activate the PS. A Colibri LED light source module (Carl Zeiss, Göttingen, Germany), which had a peak wavelength at 590–595 nm, was assembled with a customized power module and fixtures (Figure S5A). The dosimetry of the LED light source was calculated from a measurement of the intensity and power of the light using a spectroradiometer (SPR-03; Luzchem Research Inc., Ottawa, ON, Canada) (Figure S5B–D).

6.2 Evaluation of the photocytotoxic efficiency of HY-agg, HY-mPEG and HY-M

Next, 1×10^4 cells of U251MG cells were seeded in a 96-well cell culture plate (TPP Techno Plastic Products AG) with 100 μL of complete DMEM/F12 medium in quadruplicate for each DDS. After 24 h of incubation, the medium was replaced with one containing 5 μM of HY but different DDSs (HY-agg, HY-mPEG and HY-M). After 2 h of dark incubation in this medium,

the wells were washed with PBS and replaced with drug-free medium. Photodynamic therapy (PDT) using light from the LIS was performed with 5 mW/cm^2 light power (integrated over 590–605 nm) so that a total of 0.1 J/cm^2 of energy was delivered to each sample. The cells were incubated for 12 h following irradiation. Cell viability/cytotoxicity was measured using the method described above (ESI Section 5.1), and photocytotoxicity was calculated using Equation 6. All procedures involving the treatment of the HY-DDSs were performed under light-restricted conditions. The DDS-dependent photocytotoxic efficiency was calculated using Equation 7 using the photocytotoxicity and cellular uptake data (Figure S6, Table S3), which were calculated by the process described in ESI Section 5.2. Relative PEs were calculated by normalising the data for HY-mPEG and HY-M to the HY-agg results.

$$\text{Photocytotoxicity (\%)} = \left(1 - \frac{\text{measured absorbance of DDS−PDT−sample (A.U.)}}{\text{measured absorbance of control (A.U.)}}\right) \times 100 \qquad (6)$$

$$\text{Photocytotoxic efficiency (\% mg/A.U.mL)} = \frac{\text{photocytotoxicity (\%)}}{\text{cellular uptake of HY (A.U. mL/mg)}} \qquad (7)$$

6.3 Determination of the DDS-dependent co-localisation of HY in intracellular organelles

Fluorescence-based image analysis was performed to evaluate the DDS-dependent co-localisation of HY in intracellular organelles. Then 3×10^5 U251MG cells were seeded in a 35-mm culture dish (Corning Inc., Tewksbury, MA, USA) with 1 mL of complete DMEM/F12 medium. After 24 h of incubation, the cells were treated and stained with HY-DDS (5 μM, 2 h incubation) and the fluorescence probes of MitoTracker® deep red FM (200 nM, 10 min incubation), ER-Tracker™ green (1 μM, 30 min incubation) and LysoTracker® blue DND-22 (75 nM, 2 h incubation) (Molecular Probes, Eugene, OR, USA). Before imaging, the HY-DDS and dyes were washed with PBS and replaced with drug- and dye-free media. All procedures involving the treatment of the HY-DDSs were performed under light-restricted conditions. Fluorescence microscopic images of the cells were acquired with a confocal and multiphoton laser scanning microscopy imaging system (LSM510; Carl Zeiss) using a water-immersion objective lens (Achroplan 40×/0.8 numerical aperture; Carl Zeiss). The excitation light sources were 488, 543 and 633 nm continuous-wave lasers and a 800 nm femto-second pulsed laser. Emission filter sets of 390–465, 530–550, 560–615 and 650–710 nm were used. Other probes in the same field of interest were separately imaged with a single excitation laser source and a single emission filter (Figure S7). HY images were acquired last

to avoid photoactivation of HY. Imaging was done at 37°C and 5% CO_2 using an open perfusion micro-incubator (PDMI-2; Harvard Apparatus, Holliston, MA, USA).

7. Image processing and statistical analysis

7.1 Image processing

ImageJ, Adobe Photoshop CS6, Microsoft Excel and MATLAB software were used for image processing and data analysis. Co-localisation of HY in intracellular organelles (mitochondria, ER, and lysosome) was quantified with Pearson's co-localisation coefficient (Table S4). Relative co-localisation coefficients were calculated using Equation 8. The acquired images were organised using Adobe Illustrator CS6.

$$\text{Relative co} - \text{localization coefficient (A.U.)} = \frac{\text{CC of selected intracellular organelle}}{\text{sum of all CC of intracellular organelles}} \qquad (8)$$

$$\text{where CC} = \text{co-localization coefficient}$$

7.2 Statistical analysis

Statistical analysis was performed using GraphPad Prism software. Statistical differences between data were analysed using the t-test where indicated. Statistical significance was indicated when $P < 0.05$.

Supplementary Figures and Tables

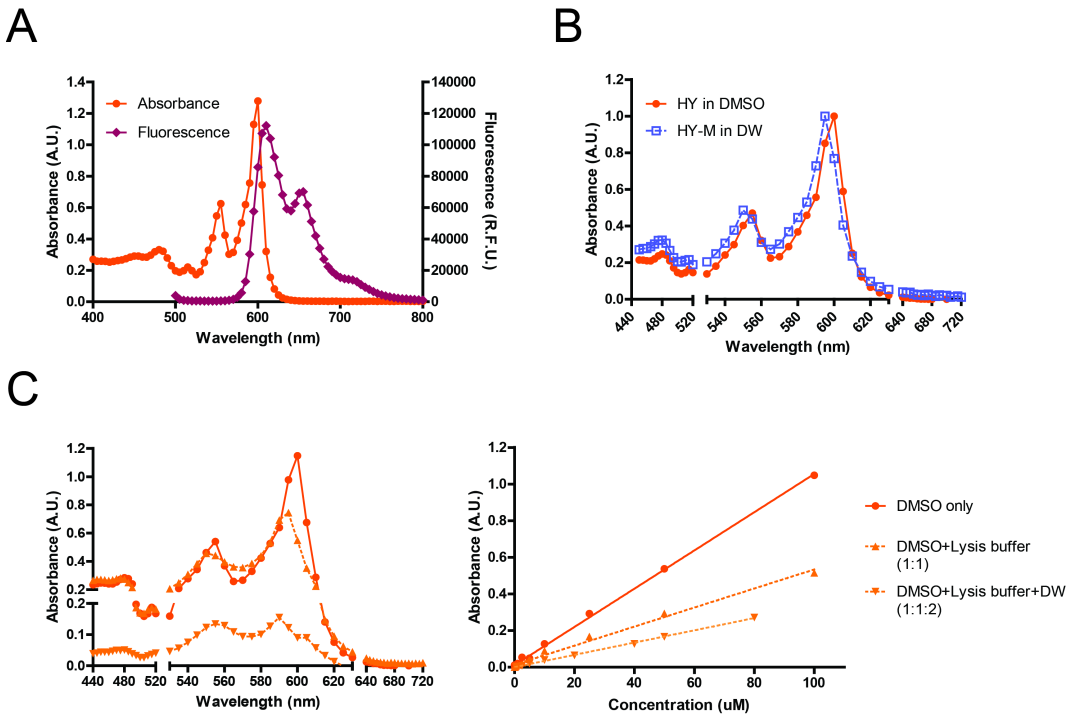

Figure S1. Absorbance and fluorescence measurements of HY. (A) Spectral absorbance and fluorescence of HY in DMSO. (B) Spectral absorbance of HY-M in DW, compared with parental HY in DMSO. (C) Spectral absorbance of HY in different solvents, and linear regression of the dose-dependent change in absorbance of HY in different solvents (at 595 nm).

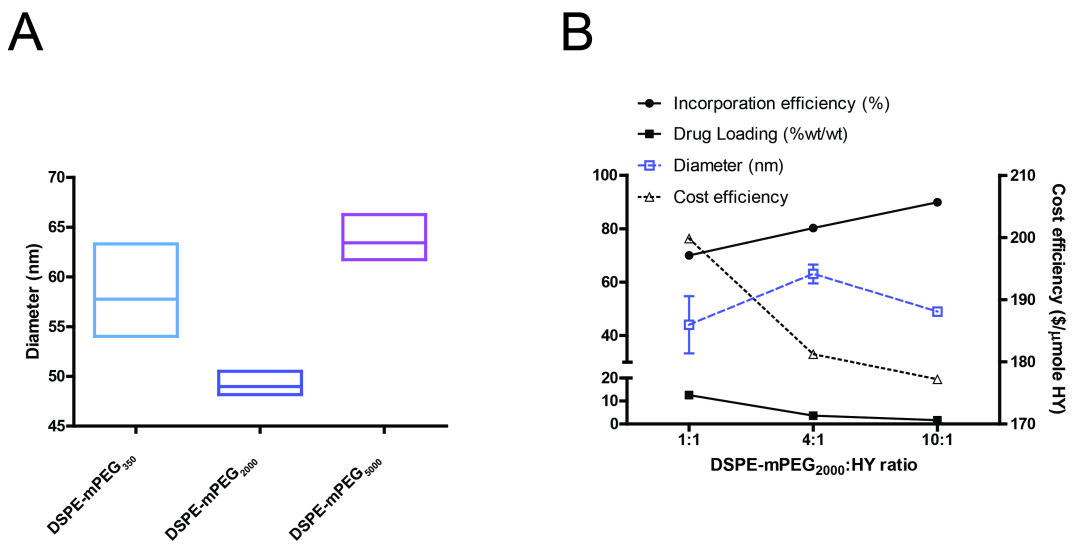

Figure S2. Characteristics of the polymeric micelles as a function of the type of polymeric lipid. (A) Number-weighted diameters of HY-Ms, which were engineered with different polymeric lipids (molar ratio of DSPE-mPEG:HY = 10:1). (B) Characteristic differences between the various HY-Ms, which were engineered with different molar ratios of DSPE-mPEG$_{2000}$:HY.

Table S1. Characteristics of the engineered micelles in DW (molar ratio of DSPE-mPEG:HY = 10:1). M_w = molecular weight; d_n = number-weighted diameter; [%]d_n = % number; Z_{av} = average zeta hydrodynamic diameter; PI = polydispersity index.

Polymeric micelles	M_w (g/mol)	Polymer concentration	Hypericin concentration	Incorporation efficiency	Drug loading (%wt/wt)	d_n (nm)	[%]d_n	Z_{av} (nm)	PI	Zeta potential (mV)
DSPE-mPEG$_{350}$	1131.5	10 mg/mL	0.2 mg/mL	87.36 %	4.46	57.77	100	108.4	0.261	-38.6
DSPE-mPEG$_{2000}$	2805.5	10 mg/mL	0.2 mg/mL	89.90%	1.80	48.97	100	111.4	0.195	-33.1

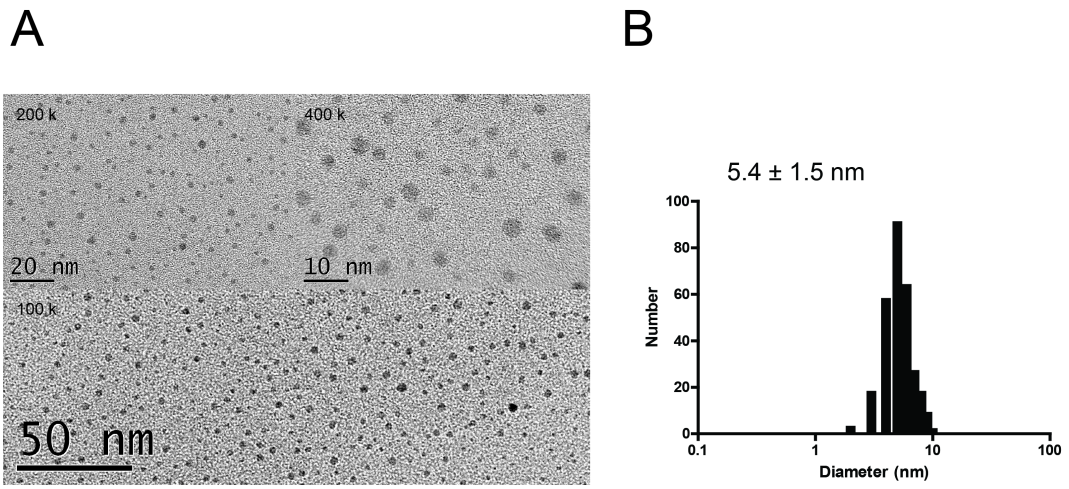

Figure S3. TEM images and measured sizes of the micelles with the non-staining method.

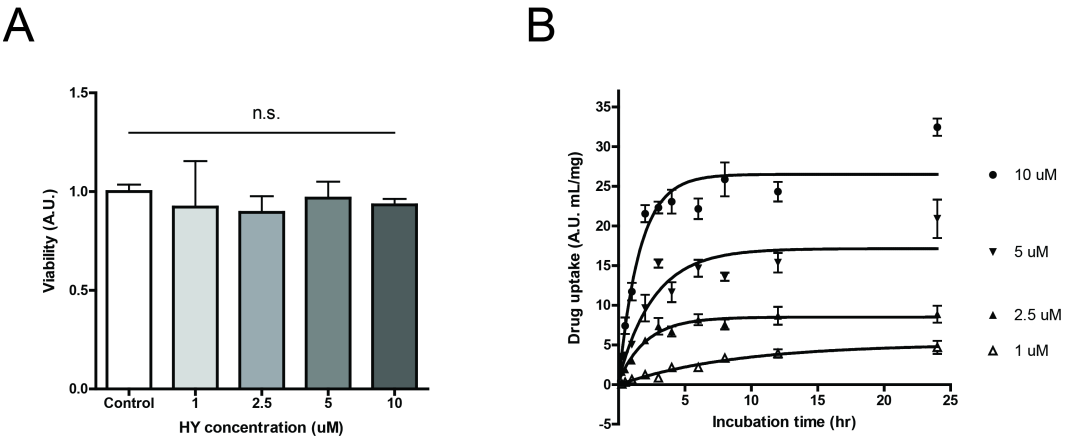

Figure S4. Cellular uptake and dark cytotoxicity determinations of HY in human glioblastoma U251MG cells. (A) Cell viability/cytotoxicity measurements with different concentrations of HY. (B) Time- and dose-dependent cellular uptake of HY.

Table S2. Best-fit values of the time- and dose-dependent cellular uptake, as calculated by the nonlinear one phase association regression model. Y_0 = Y value when X (time) is zero; Plateau = Y value at infinite times; K = rate constant; τ = time constant; Half-time = $\frac{\ln 2}{K}$; Span = difference between Y_0 and the plateau.

HY concentration (μM)	Y_0	Plateau	K	τ	Half-time (hour)	Span
1	-0.0348	5.105	0.1141	8.762	6.073	5.14
2.5	0.3894	8.499	0.4758	2.102	1.457	8.11
5	0.5627	17.13	0.3728	2.682	1.859	16.57
10	-0.01962	26.5	0.6324	1.581	1.096	26.52

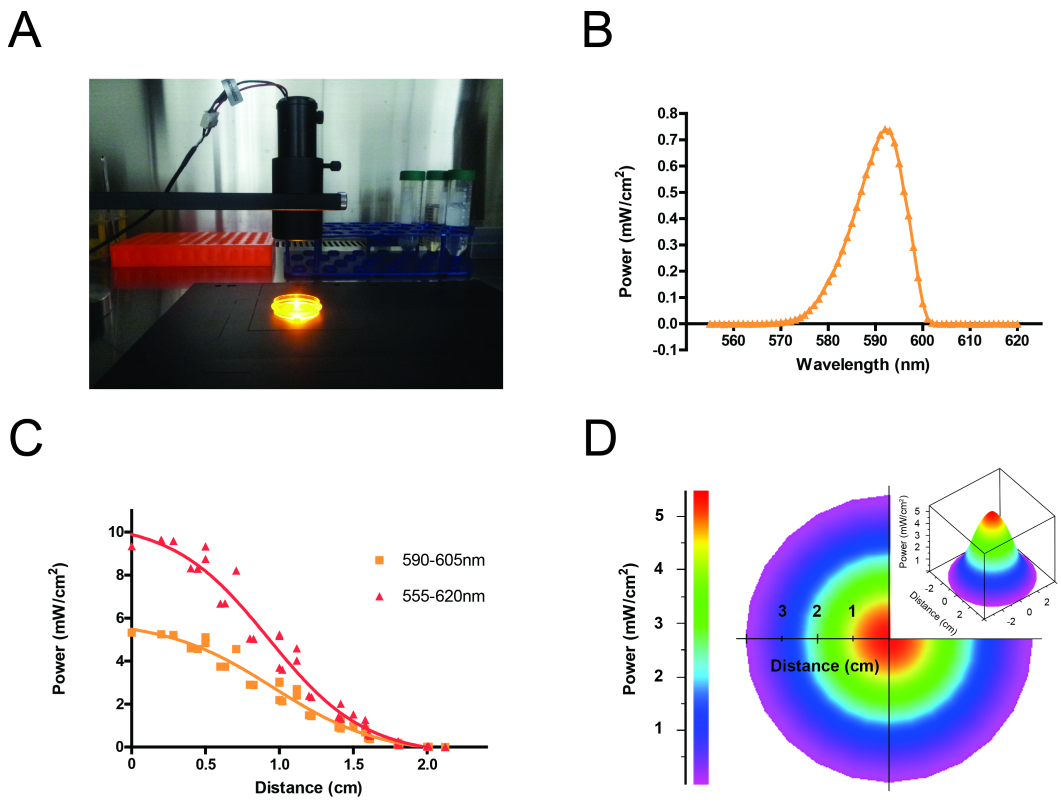

Figure S5. LED-based LIS and dosimetry. (A) Photograph of the LED-based LIS. (B) Wavelength- dependent power spectrum of the LED light. (C-D) Distance-power map of the LED-based LIS.

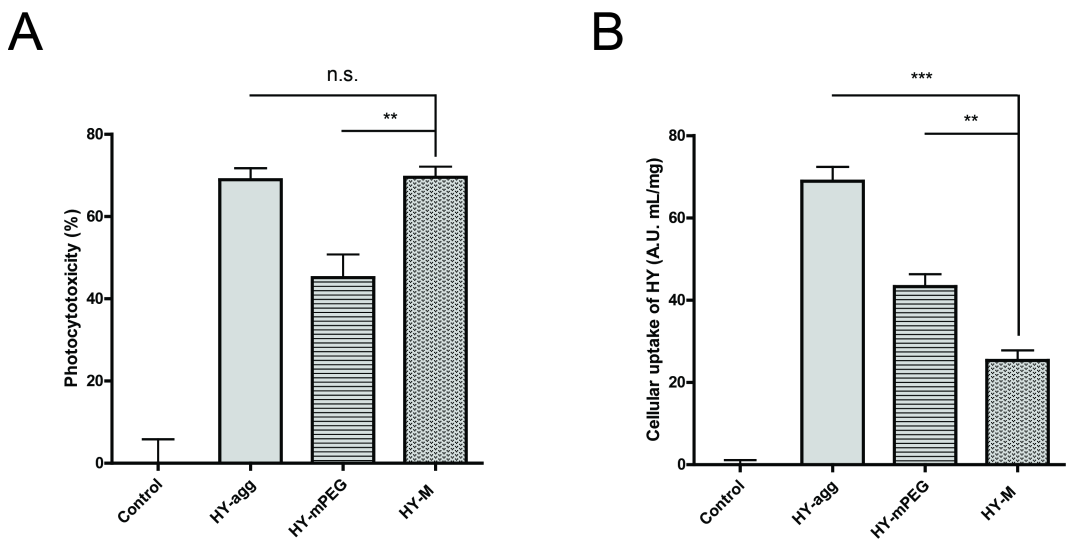

Figure S6. Photocytotoxicity (A) and cellular uptake (B) of HY in all HY formulations (HY-agg, HY-mPEG and HY-M). **, $P < 0.01$; and ***, $P < 0.001$.

Table S3. Values of the photocytotoxicity and cellular uptake of HY in all HY formulations (mean ± standard error of mean).

	Control	**HY-agg**	**HY-mPEG**	**HY-M**
Photocytotoxicity (%)	0.00 ± 5.83	68.91 ± 2.83	45.12 ± 5.64	69.54 ± 2.57
Cellular uptake of HY (A.U. mL/mg)	0.00 ± 1.11	68.91 ± 3.47	43.31 ± 3.02	25.33 ± 2.47

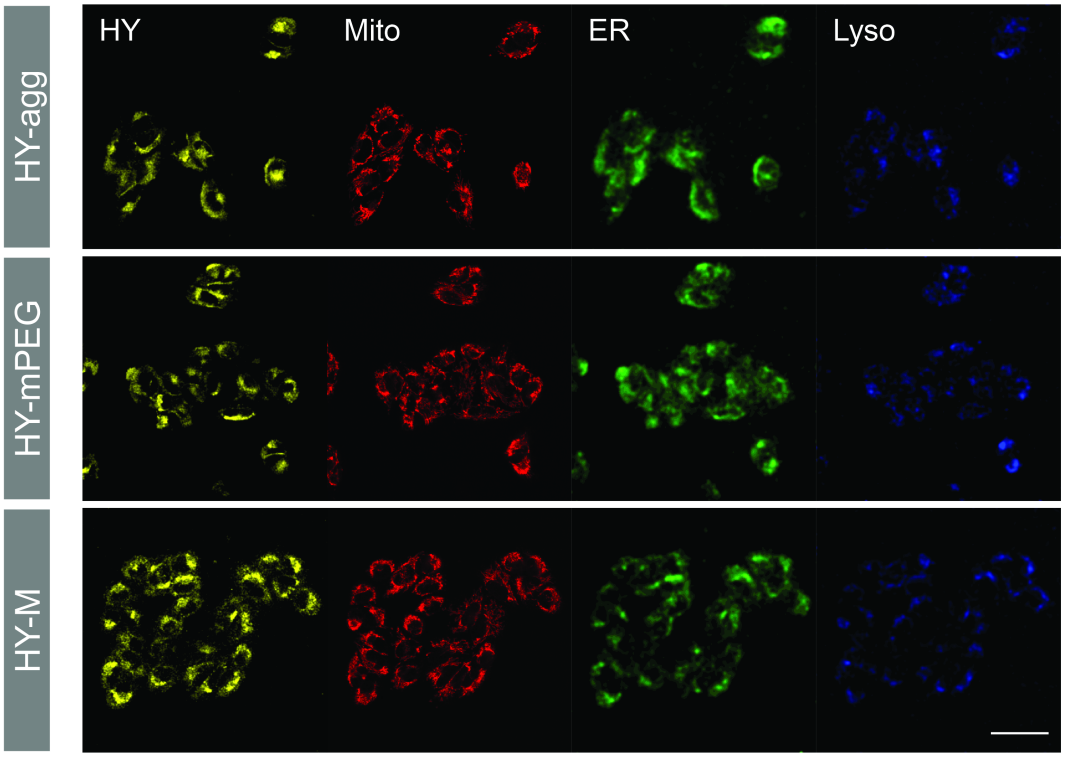

Figure S7. DDS-dependent co-localisation of HY in intracellular organelles of human glioblastoma U251MG cells. The images were acquired by confocal and multiphoton laser scanning microscopy.

Table S4. DDS-dependent relative co-localisation coefficients of HY in intracellular organelles (mean ± standard error of mean). ***, $P < 0.001$ (compared to two other DDSs).

	HY-Mitochondria	HY-Endoplasmic reticulum	HY-Lysosome
HY-agg	0.555 ± 0.013	0.332 ± 0.010	0.113 ± 0.004
HY-mPEG	0.543 ± 0.022	0.347 ± 0.014	0.110 ± 0.006
HY-M	0.635 ± 0.012***	0.252 ± 0.017***	0.113 ± 0.007

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