Electronic Supplementary Material (ESI) for Chemical Communications
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Supplementary Information

Experimental Methods

Materials

Fullerene (C_{60}) was purchased from NanoLab Inc. (Waltham, MA, USA). Sodium hyaluronate (HA, *M*w = 4 kDa), lithium hydroxide (LiOH), hyaluronidase type 2 (Hyal-2), dimethyl sulfoxide (DMSO), and 9,10-dimethylanthracene (DMA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI 1640, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Welgene Inc (Seoul, South Korea). The Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Molecular Technologies Inc (Kumamoto, Japan).

Synthesis of HA-g-C_{60}

As shown in Scheme 1 and Table 1, C_{60} was grafted to HA using LiOH as a catalyst in DMSO at room temperature for 2 days. After the reaction, the resulting solution was transferred to a pre-swollen dialysis membrane tube (Spectra/Por® MWCO 15K) and was dialyzed against borate buffer (pH 7.4) solution to remove non-reacted chemicals. The solution withdrawn from a dialysis membrane tube was lyophilized after freeze-drying for 2 days. The conjugation of C_{60} to HA was estimated from ^{1}H NMR (DMSO-d_6 with TMS) from the peaks from δ 7.25 ppm [-C**H** in C_{60} part of HA-*g*-C_{60}] and δ 2.30 ppm [-C$\mathbf{H}_3$ in HA] (Supplementary Fig. 1).

Characterization of HA-g-C_{60}

The particle size distribution of HA-*g*-C_{60} (1 mg/ml) in 150 mM PBS (pH 7.4) was measured with a Zetasizer 3000 instrument (Malvern Instruments) equipped with a He-Ne Laser beam at a wavelength of 633 nm and a fixed scattering angle of 90°. The morphology of HA-*g*-C_{60} (10 μg/ml) was confirmed using a field emission scanning electron microscopy (FE-SEM, Hitachi s-4800, Tokyo, Japan). Optical images from vials containing HA-F1 (1 mg/1 ml, equivalent C_{60} 0.05 mg/ml), HA-F3 (1 mg/1 ml, equivalent C_{60} 0.20 mg/ml), and HA-F5 (1 mg/1 ml, equivalent C_{60} 0.40 mg/ml) were obtained in PBS (pH 7.4). The UV/visible spectra of HA-*g*-C_{60} conjugates (0.1 mg/ml) and free C_{60} (0.1 mg/ml) in 150 mM PBS (pH 7.4) were monitored at 400–800 nm. The self-quenching effect of HA-*g*-C_{60} conjugates (0.1 mg/ml) was analyzed in DMSO with a KODAK image station ($\lambda_{excitation}$ = 635 nm, $\lambda_{emission}$ = 710 nm). The generation of singlet oxygen of HA-*g*-C_{60} (equivalent C_{60} 0.1 mg/ml) or free C_{60} (0.1 mg/ml) in 150 mM PBS (pH 7.4) or DMSO was confirmed using 9,10-dimethylanthracene (DMA). DMA (20 mmol) was mixed with HA-*g*-C_{60} (equivalent C_{60} 0.1 mg/ml) in 150 mM PBS (pH 7.4) or DMSO. The solution was illuminated at a light intensity of 10 mW/cm^2 using a 670 nm laser source for 5 min. When the DMA fluorescence intensity (measured using a Shimadzu RF-5301PC spectrofluorometer at λ_{ex} 360 nm and λ_{em} 380-550 nm) reached a plateau after 1 h, the change in DMA fluorescence intensity (F_f - F_s) was plotted after subtracting each sample fluorescence intensity (F_s) from the full DMA fluorescence intensity (without HA-*g*-C_{60} or C_{60}, indicating no singlet oxygen, F_f). Particle size distribution of HA-*g*-C_{60} conjugates (0.1 mg/ml) after incubation with 0.1-5 mg/ml of hyaluronidase type 2 (Hyal-2) at 37 °C for 1 h were analyzed using Zetasizer 3000 instrument (Malvern Instruments). In addition, the specific molar extinction coefficient of HA-F1 (euqivalent equivalent C_{60} 0.1 mg/ml) or free C_{60} (0.1 mg/ml) in PBS (pH 7.4) was determined at 635 nm wavelength by using the Lambert-Beer's equation. The specific molar extinction coefficient of HA-F1 or free C_{60} was 1.09×10^4 or 60.14, respectively.

In vitro phototoxicity

Human colon carcinoma HCT-116 cells and human nasopharyngeal epidermal carcinoma KB cells (from Korean Cell Line Bank) were maintained in RPMI 1640 medium with 2 mM $_L$-glutamine, 1 % penicillin-streptomycin, and 10 % FBS in a humidified standard incubator with a 5 % CO_2 atmosphere at 37 °C. Prior to testing, cells (1×10^5 cells/ml), grown as a monolayer, were harvested via trypsinization using a 0.25 % (w/v) trypsin/0.03% (w/v) EDTA solution. The cells suspended in RPMI-1640 medium were seeded onto well plates and cultured for 24 h prior to *in vitro* cell testing.

Phototoxicity of HA-*g*-C_{60} with light illumination was tested in HCT-116 (with CD44 receptor) and KB tumor cells (without CD44 receptor). HA-*g*-C_{60} or free C_{60} dispersed in RPMI 1640 medium was administered to cells plated in 96-well plates. The cells were incubated with each sample for 4 h and then washed three times with 150 mM PBS (pH 7.4). The cells were illuminated at a light intensity of 10 mW/cm^2 using a 670 nm laser source for 5 min and then further incubated for 12 h. Cell viability was determined using a Cell Counting Kit-8 (CCK-8 assay) (n=7).

In addition, for evaulating the competition effect by free HA, HA-*g*-C_{60} (equivalent C_{60} 1 μg/ml) dispersed in RPMI 1640 medium with 100 μg/ml of free HA was administered to cells plated in 96-well plates. The cells were incubated with each sample for 4 h and then washed three times with 150 mM PBS (pH 7.4). The cells were illuminated at a light intensity of 10 mW/cm^2 using a 670 nm laser source for 5 min and then further incubated for 12 h. Cell viability was determined using a CCK-8 assay (n=7).

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Furthermore, the cell viability test of HCT-116 and KB cells treated with HA-*g*-C_{60} (equivalent C_{60} 1-50 μg/ml) without light illumination was conducted to estimate the original toxicity of HA-*g*-C_{60}. The cells were incubated for 24 h with HA-*g*-C_{60} and then evaluated via a CCK-8 assay.

The tumor cellular uptake of HA-*g*-C_{60} (1 μg/ml) was monitored using a confocal microscope ($\lambda_{excitation}$ = 635 nm, $\lambda_{emission}$ = 710 nm). The cells were incubated with each sample for 4 h and then washed three times with 150 mM PBS (pH 7.4). During microscope observation, to ameliorate any fluorescence photobleaching, a drop of anti-fade mounting solution (5% N-propyl galate, 47.5% glycerol and 47.5% Tris–HCl, pH 8.4) was added to the cells (Supplementary Fig. 4).

Transmission electron microscopy (TEM)

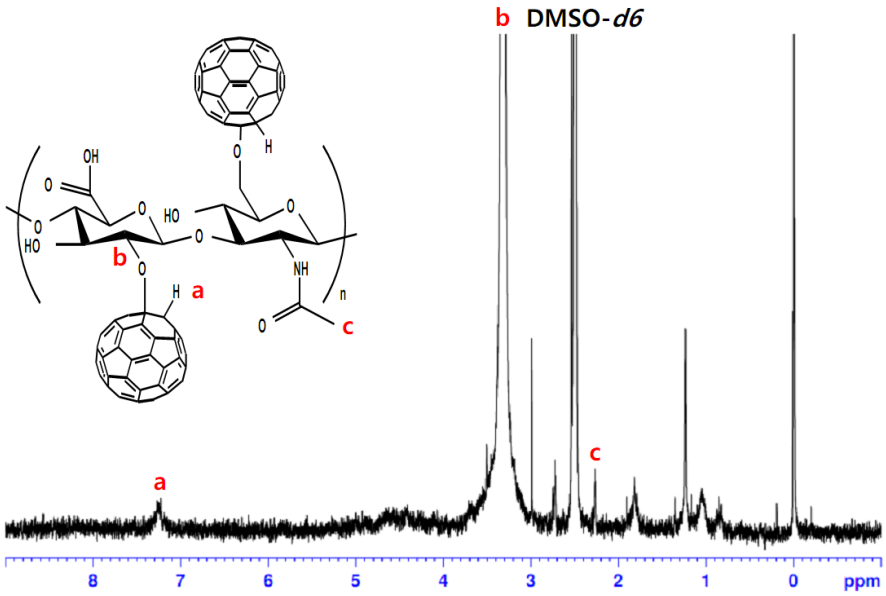
The tumor cells, incubated with HA-*g*-C_{60} (equivalent C_{60} 5 μg/ml) for 2-6 h at 37 °C, were washed three times with fresh PBS and fixed in 4 % paraformaldehyde/2.5 % glutaraldehyde in 0.1 M PBS for overnight. After washing with 0.1 M PBS, the specimens were postfixed with 1 % osmium tetroxide in PBS for 1 h. The specimens were then dehydrated with pure ethyl alcohol or acetone and embedded in Epon 812 (embedding resin). The polymerization of Epon 812 was performed at 60 °C for 3 days. Ultra-thin sections (60-70 nm) from embedded specimens were obtained using ultra-microtome (Leica Ultracut UCT, Germany). Ultra-thin sections were mounted onto carbon-coated copper grids and examined using TEM (JEM 1010, Japan) operating at 60 kV and CCD camera (SC1000 Orion, USA)

Animal care

In vivo studies were conducted with 4 to 6-week old female nude mice (BALB/c, nu/nu mice, Institute of Medical Science, Tokyo, Japan). Mice were maintained under the guidelines of an approved protocol from the Institutional Animal Care and Use Committee (IACUC) of the Catholic University of Korea (Republic of Korea).

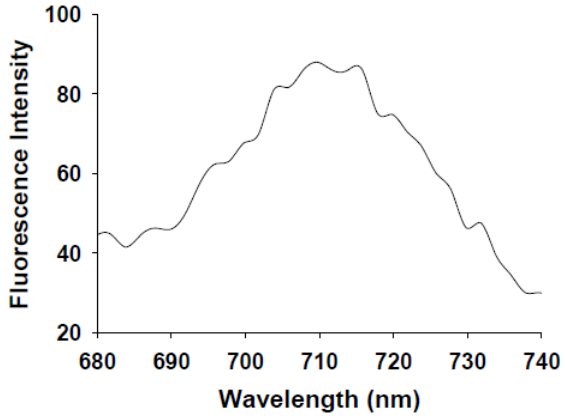
In vivo photoluminescent imaging and tumor inhibition

For the *in vivo* animal experiments, HCT-116 and KB tumor cells were introduced into female nude mice via subcutaneous injection of 1×10^4 cells suspended in PBS pH 7.4 (ion strength: 0.15) medium. When the tumor volume reached about 40 mm^3, HA-*g*-C_{60} conjugates (equivalent C_{60} 10 mg/kg body) or PBS solution (control, 150 mM, pH 7.4) was injected intravenously into tumor-bearing nude mice through the tail vein. A 12-bit CCD camera (Image Station 4000 MM; Kodak, Rochester, NY, USA) prepared with a special C-mount lens and a long wave emission filter (710 nm; Omega Optical, Brattleboro, VT, USA) were used to capture live photoluminescent images of the nude mice. Additionally, 24 h after the injection, the tumor sites of nude mice were locally irradiated for 40 min at a light intensity of 10 mW/cm^2 with a 670 nm laser source. The change in tumor volume was monitored over elapsed time. Tumor volume was calculated using the formula: tumor volume=length×$(width)^2/2$. Additionally, the change in the body weight of treated nude mice proved insignificant (data not shown) and their vital behaviors appeared equivalent to the non-treated nude mice, suggesting a low possibility in side effects. All results were analyzed via Student's t-test or ANOVA and null hypotheses of no difference were rejected if p-values were less than 0.05. MINITAB® *release 14* statistical software was used.



Supplementary Fig. 1. ^{1}H NMR peak of hyaluronated fullerene (HA-F1)

Supplementary Fig. 2. Field emission scanning electron microscope (FE-SEM) images of HA-F1 (left), HA-F3 (middle), and HA-F5 (right)



Supplementary Fig. 3. The emission spectra of HA-F1 (1 mg/ml) in PBS (150 mM, pH 7.4) after excitation at 635 nm.

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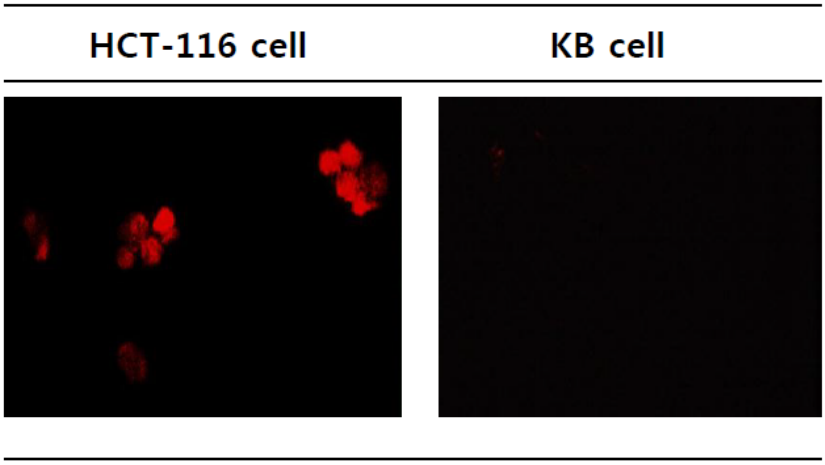
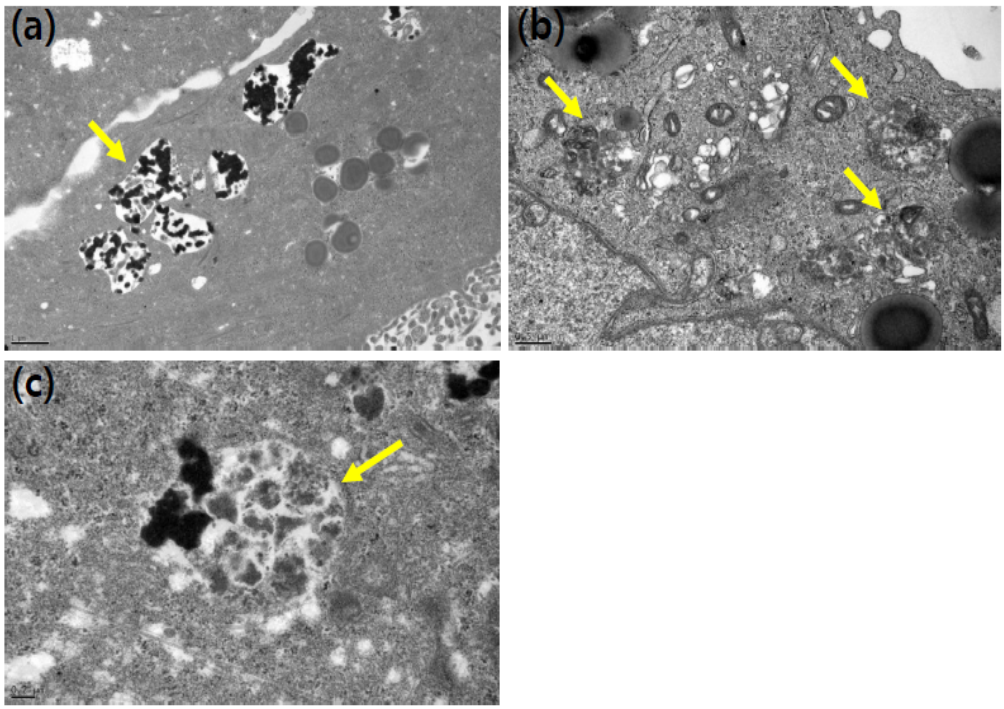
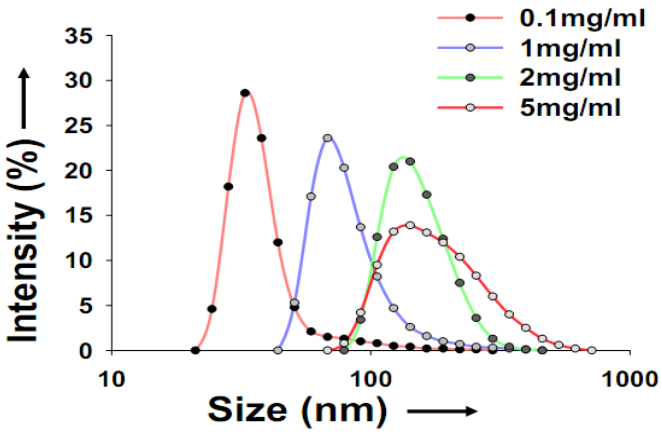


Supplementary Fig. 4. Cellular uptake of HA-F1 in HCT-116 or KB cells treated with HA-F1 (1μg/ml) for 4 h. Red fluorescence ($\lambda_{excitation}$ = 635 nm, $\lambda_{emission}$ = 710 nm) depicts the presence of HA-F1.



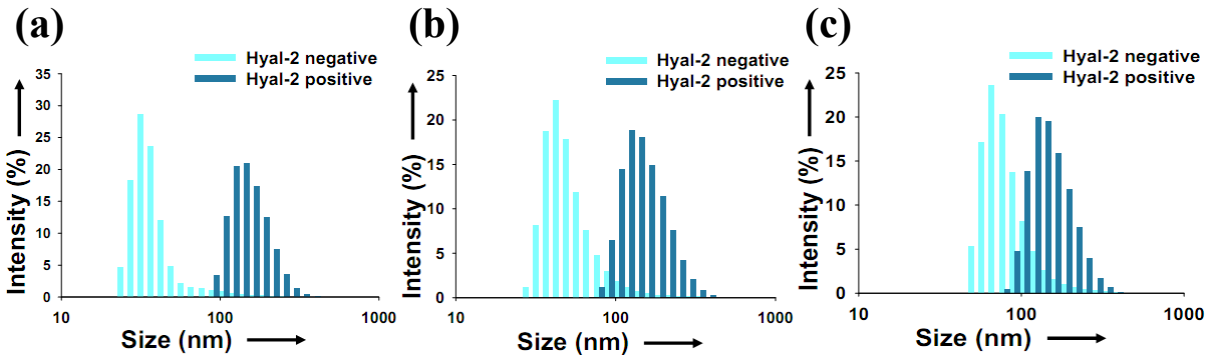
Supplementary Fig. 5. TEM analysis of HCT-116 tumor cells treated with HA-*g*-C_{60} (equivalent C_{60} 5 μg/ml) for (a) 2 h (1 μm bar scale in TEM image) and (b) 6 h (0.5 μm bar scale in TEM image) at 37 °C. (c) shows the enlarged TEM image (0.2 μm bar scale in TEM image) of (b). Endocytosed HA-*g*-C_{60} conjugates are indicated by the yellow arrows.

The TEM images reveal that endocytosed HA-*g*-C_{60} conjugates were efficiently degraded by Hyal-2 in HCT-116 cells.



Supplementary Fig. 6. Particle size change of HA-F1 after incubation with 0.1-5 mg/ml of hyaluronidase type 2 (Hyal-2) for 1 h.

HA can be specifically degraded by hyaluronidase (HYAL) such as Hyal-1 and Hyal-2. To evaluate enzyme degradability of HA-*g*-C_{60} conjugates, we used Hyal-2 which is a prevalent protein that initiates the cleavage of HA. When incubated with Hyal-2 at 1 mg/ml concentration for 1 h, particle sizes of HA-*g*-C_{60} conjugates (HA-F1, HA-F3, and HA-F5) rapidly increased, and particle sizes of HA-F1 became significantly larger when the concentration of Hyal-2 increased from 0.1 to 5 mg/ml, implying the collapse of HA-*g*-C_{60} nanoparticles (resulting in producing large C_{60} aggregates due to poor solubility of C_{60} molecules) owing to degradation of the HA backbone by Hyal-2.



Supplementary Fig. 7. Particle size change of (a) HA-F1, (b) HA-F3, and (c) HA-F5 after incubation with 1 mg of hyaluronidase type 2 (Hyal-2) (Hyal-2 positive) or without Hyal-2 (Hyal-2 negative) for 1 h.