Electronic Supplementary Information

Facile Construction of Well-defined Fullerene-Dendrimer Supramolecular Nanocomposites for Bioapplications

Xiaojie Li,[a] Yasuo Watanabe,[a] Eiji Yuba,[a] Atsushi Harada,[a] Takeharu Haino [b] and Kenji Kono*[a]

[a] Department of Applied Chemistry, Graduate School of Engineering, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan.

[b] Department of Chemistry, Graduate School of Science, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima City 739-8526, Japan.

*Correspondence to: kono@chem.osakafu-u.ac.jp

1. Materials

Fullerene (C_{60}) was obtained from Nacalai Tesque (Japan). Diethyl malonate, tetrabromomethane (CBr_4), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), dimethyl sulfoxide (DMSO), and 3-(4,5-dimethyl-2-thiazoryl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Wako Pure Chemical (Osaka, Japan). Generation 4 PAMAM dendrimer (10 wt.% in methanol) and generation 5 PAMAM dendrimer (5 wt.% in methanol) were obtained from Sigma-Aldrich Japan (Tokyo, Japan). Tetrahydrofuran (THF), toluene, sodium hydride (NaH), and methanol (CH_3OH) were supplied from Kishida Chemical (Osaka, Japan). PEG 4-nitrophenyl carbonate (PEG-*NPC*) was synthesized using 4-nitrophenyl chloroformate (Tokyo Chemical Industry) and polyethylene glycol monomethyl ether (average Mn ~2000, Sigma-Aldrich) according to our previous report.[1] Fluorescein isothiocyanate (FITC)-labelled generation 4 PAMAM dendrimer (FI-PAMAM) and ethoxy diethylene glycol-modified generation 4 PAMAM dendrimer (EDEG-G4) named as oligo(ethylene glycol)-modified generation 4 PAMAM dendrimer (OEG-G4) in the present study was prepared according our previous report.[2] THF, toluene and DMSO were distilled just prior to use. Other reagents without statement were used as received.

2. Preparation and characterization of fullerene derivatives.

Malonic acid fullerene derivatives were prepared according to a previously reported method.[3] Briefly, C_{60} fullerene was converted into fullerene malonic ester by reaction with diethyl malonate, CBr_4 and DBU in dry toluene at dark. The mono-malonate fullerene and di-malonate fullerene were successfully obtained through silica gel column chromatography separation and verified by characterization of ^{13}C NMR (JNM-LA-400, JEOL, Japan) and MALDI-TOF-MASS spectra (KOMPACT PROBE/MALDI 2, Shimadzu Corp., Japan). The theoretical molecular mass of mono-malonate fullerene and di-malonate fullerene were [M] = 878.79 and 1036.95, respectively, and the MALDI-TOF-MASS result shows $[M + H^+]$ = 879.1 and 1038.8, respectively. Then, the obtained malonate fullerenes were treated with NaH followed by CH_3OH in toluene for ester hydrolysis, and finally with HCl to give the final malonic acid fullerenes, as confirmed by Fourier transform infrared spectroscopy (FTIR-8400, Shimadzu Corp., Japan).

3. Preparation of PEG-modified PAMAM dendrimers.

PEG-modified generation 4 PAMAM dendrimer (PEG-G4) was prepared according to our previous report.[1] FITC-labeled PEG-modified generation 4 PAMAM dendrimer (FI-PEG-G4) was synthesized using the same method by reaction of PEG-*NPC* and FI-PAMAM.[1,2] PEG-modified generation 5 PAMAM dendrimer (PEG-G5) was also synthesized according to the same method except for the amount of the reagents used for the preparation. Briefly, PEG-*NPC* (900 μmol) was reacted with generation 5 PAMAM dendrimer (3.5 μmol) in DMSO, followed by purification using a Sephadex G-75 column (Pharmacia) and dialysis. Yield: 731.6 mg (27.7%). ^1H NMR (D_2O, JNM-LA-400, JEOL, Japan): PAMAM: δ 2.07 (br), 2.28 (br), 2.47 (br), 2.90 (m), 2.96 (m); PEG: δ 3.05 (s), 3.56 (m), 3.86 (br).

4. Preparation of fullerene-dendrimer supramolecular nanocomposites.

Fullerene-dendrimer supramolecular nanocomposites were prepared according to our previously reported method.[4] Briefly, dendrimers (PEG-G4, or PEG-G5, or OEG-G4,

3.25 nmol) and fullerenes (65 to 357.5 nmol) at varying fullerene/dendrimer feed ratios were dissolved in 2 mL THF (for mono- and di-malonic acid fullerenes) or toluene (for unmodified fullerene and mono-malonate fullerene), and incubated for 1 h at 25 °C. The solvent was then evaporated under reduced pressure to form a dry film. The resulting mixture was re-dispersed in 3 mL distilled water, and the precipitates of free fullerenes were removed by a 10 min centrifugation at 10000 rpm, affording the aqueous solution of fullerene-dendrimer supramolecular nanocomposites. Typically, 2 mL of supernatant was lyophilized and re-dissolved in THF or toluene, and the extraction efficiency of fullerenes was determined by measuring the absorbance at 298 nm using a UV-visible spectrophotometer (V-560, JASCO Inc., Japan).

5. General characterizations for MF_{60}-PEG-G4 supramolecular nanocomposites.

The formation of MF_{60}-PEG-G4 nanocomposites was examined by Gel Permeation Chromatography (GPC, TSK-gel G3000PW and G4000PW; Toso Co., Ltd., Japan) equipping with two detectors, a differential refractive index detector (RI-930; Jasco Inc., Japan) and an UV−vis detector (λ = 426 nm; UV-970; Jasco Inc., Japan). The aqueous solutions of PEG-G4 and MF_{60}-PEG-G4 nanocomposites were prepared at a concentration of 2 mg/mL, and 10−20 μL of these sample solutions were injected. The samples were eluted with 10 mM phosphate buffer containing 0.2 M sodium sulfate solution (pH 6.24) at 0.5 mL/min at 30 °C. The particle size of PEG-G4 and MF_{60}-PEG-G4 nanocomposites in PBS at a dendrimer concentration of 0.2 mg/mL were measured by dynamic light scattering (DLS) carried out on a Zetasizer Nano ZS90 (Malvern Instruments) with a standard He−Ne 633 nm laser and 173° back scatter. The data were analyzed by Malvern Dispersion Technology Software 7.02. Atomic force microscopy (AFM) measurements (dynamic force mode) were performed by SPI3800 probe station and SPA400 unit system (Seiko Instruments Inc.). The cantilever was made of silicon (SI-DF40; Seiko Instruments Inc.), and its spring constant was 16 N/m. Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL JNM-LA 400 instrument. MF_{60}-PEG-G4 nanocomposites were prepared at a fullerene/dendrimer feed ratio of 100. The nanocomposites were then freeze-dried and redissolved in D_2O for ^1H NMR

analysis. Fluorescence spectra of MF_{60}-FI-PEG-G4 nanocomposites prepared at a fullerene/dendrimer feed ratio of 20 was measured by excitation at 488 nm using a spectrofluorometer (Jasco FP-6500).

6. *In vitro* stability study of carboxyfullerenes-dendrimer supramolecular nanocomposites.

The stability of carboxyfullerenes-dendrimer nanocomposites in PBS was examined by a dialysis method. Briefly, 3 mL of MF_{60}-PEG-G4 or DF_{60}-PEG-G4 nanocomposites prepared at a carboxyfullerene/PEG-G4 feed ratio of 20 was dissolved in PBS at the dendrimer concentration of 2.0 mg/mL, and was dialyzed against 300 mL PBS at 37 °C using a dialysis tube (cutoff molecular weight 12000-14000, Seamless cellulose tubing). The external buffer solution was replaced with 300 mL fresh PBS every 3 h. Periodically, the residues in the dialysis bag were diluted 20 times by THF, and the time course retention of carboxyfullerenes in PEG-G4 dendrimers were determined by measuring the absorbance at 298 nm in THF.

7. Cell culture.

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO), supplemented with 10% fetal bovine serum (FBS, GIBCO), penicillin (50 units/mL) and streptomycin (50 μg/mL) at 37 °C under 5% CO_2 condition. Cells were cultured for 2 days to achieve approximately same confluence before performing all experiments.

8. Confocal laser scanning microscopy.

MF_{60}-FI-PEG-G4 nanocomposites were prepared at a fullerene/dendrimer feed ratio of 20. The nanocomposites were then freeze-dried and redissolved in PBS with a final dendrimer concentration of 2 mg/mL. HeLa cells (2×10^5 cells) were seeded into a 35 mm dish in 2 mL of DMEM supplemented with 10% FBS, and cultured for 24 h. After washing twice with PBS, 0.5 mL of MF_{60}-FI-PEG-G4 nanocomposites solution and 1.5 mL DMEM medium were added. After incubation for 5 h, cells were gently washed

with PBS twice, and incubated for another 0, or 19 h in 2 mL of DMEM supplemented with 10% FBS, followed by addition of 30 μL of LysoTracker Green DND-26 (10 pmol/μL in water) and 2 μL of Hoechst33342 (10 mg/mL in water). After 30 min staining, cells were washed with PBS three times. Confocal laser scanning microscopic (CLSM) analysis of these cells was performed on an LSM 5 EXCITER (Carl Zeiss Co. Ltd.).

9. *In vitro* cytotoxicity assay.

MF_{60}-PEG-G4 nanocomposites prepared at a fullerene/dendrimer feed ratio of 20 were used. HeLa cells were seeded into a 24-well microplate (5×10^4 cells/well) in 500 μL of DMEM supplemented with 10% FBS, and cultured for 24 h. After washing twice with PBS, 500 μL of DMEM supplemented with 10% FBS along with 20 μL of MF_{60}-PEG-G4 nanocomposites at various concentrations in PBS (pH 7.4) per well were added. After incubated with sample solutions for 5 h, cells were gently washed with PBS twice, and irradiated with light ($\lambda = \sim 530$ nm) using a super high pressure mercury lamp (USH-500D, USHIO) equipped with a light filter (G-53S, Toshiba) for 3 h in PBS, followed by 20 h incubation in 500 μL of DMEM supplemented with 10% FBS. Then, the cell viabilities were determined by MTT assay. DMEM (470 μL) and 30 μL MTT solution (10 mg/mL in PBS) were added as the incubation media. After 3 h, MTT media were removed, and cells were washed twice with PBS. Then, 450 μL of isopropanol with 50 μL of 1 N HCl solution was added, and absorbance at 490 nm was measured on a VICTOR(3) V 1420 Multilabel Counter (PerkinElmer, USA) to check the cells' surviving profile.

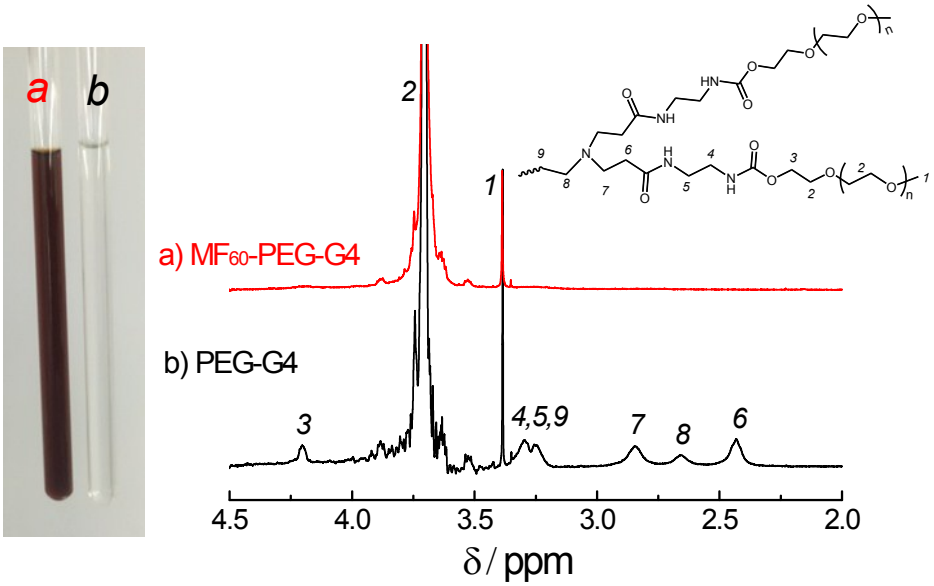

Figure S1. ^{1}H NMR spectra of MF_{60}-PEG-G4 and PEG-G4 recorded in D_2O. A photograph of NMR tubes containing MF_{60}-PEG-G4 (a) and PEG-G4 (b) in D_2O is also shown.

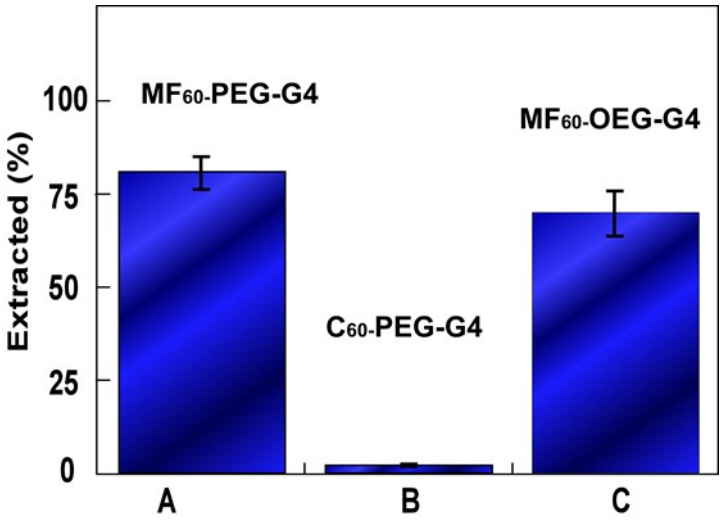

Figure S2. Supramolecular nanocomposites formation of MF_{60} with PEG-G4 dendrimer and OEG-G4 dendrimer prepared at a fullerene/dendrimer feed ratio of 40. C_{60} was used as a control.

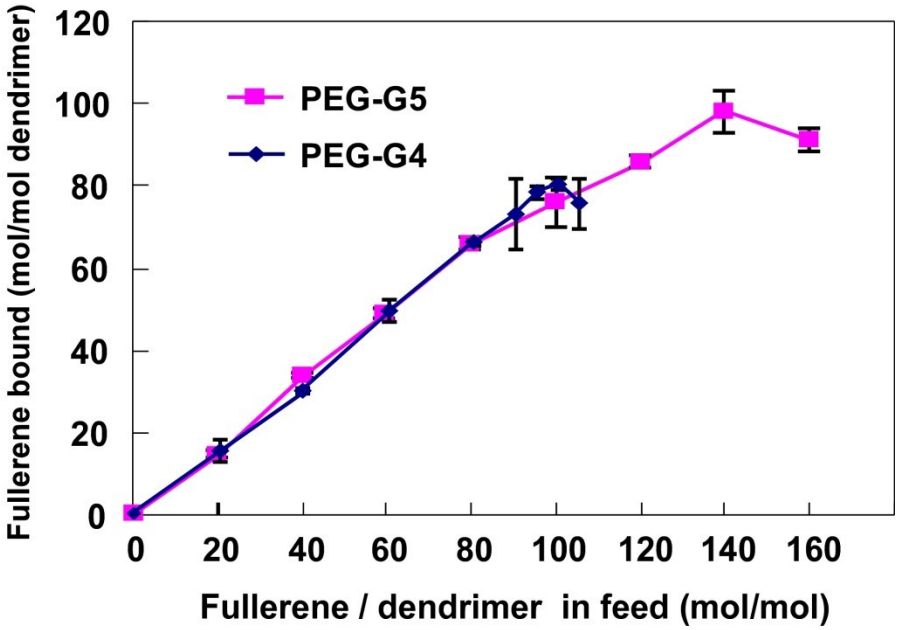

Figure S3. Supramolecular nanocomposites formation of MF_{60} with PEG-G4 dendrimer and with PEG-G5 dendrimer at varying fullerene/dendrimer feed ratios.

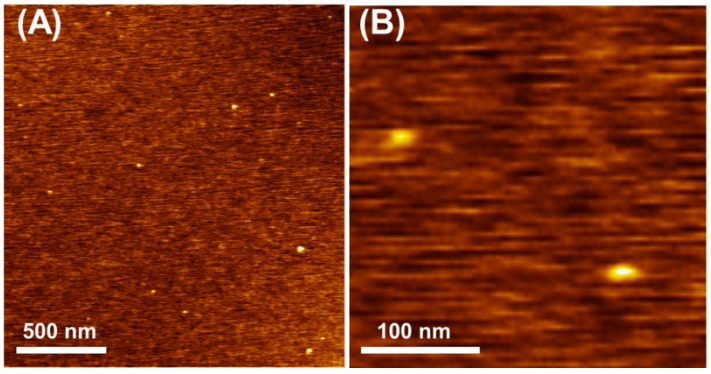

Figure S4. AFM image of the empty PEG-PAMAM-G4 dendrimer (A) and its higher magnification image (B).

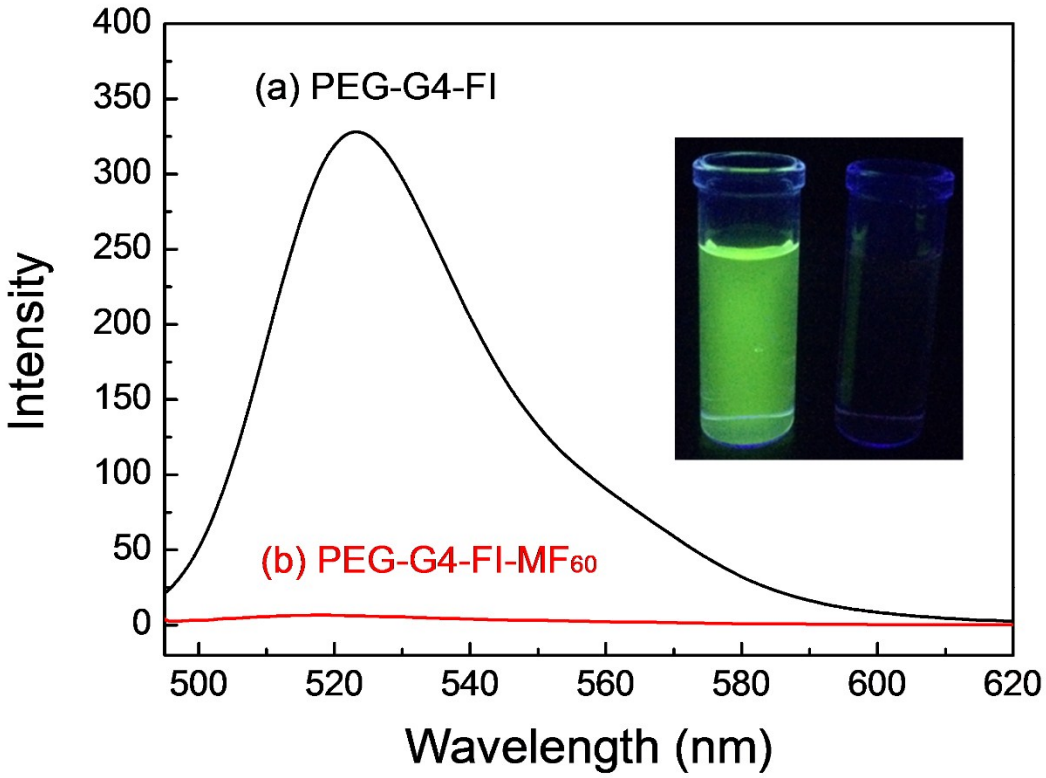

Figure S5. Fluorescence spectra of MF_{60}-FI-PEG-G4 and FI-PEG-G4 aqueous solutions (0.1 mg/mL). Photographs for FI-PEG-G4 (left) and MF_{60}-FI-PEG-G4 (right) aqueous solutions (0.5 mg/mL) under 365 nm UV light irradiation are also shown.

References

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