Note

Synthesis, Aggregation Behavior, and Photodynamic Properties of a Water-Soluble Fulleropyrrolidine Bearing an *N*-PEG Pyridinium Unit

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Herein, we describe the synthesis of a water-soluble photodynamically active fullerene bearing a polyethylene glycol chain and a hydrophilic cationic group, revealing that the solubility of the above derivative in aqueous medium depends on ultrasonication time, with the particle size of aggregates being correlated with concentration.

Key words fullerene derivative; polyethylene glycol; photodynamic activity

Nano-size carbon materials can potentially be applied in various fields, particularly in biochemistry and biomedicine.^{1–5)} For example, fullerene C₆₀ is known to exhibit unique antibacterial,^{6,7)} antitumor,^{8,9)} and antioxidant activity.¹⁰⁾ However, in order for these properties to be efficiently utilized, C₆₀ derivatives need to be water-soluble. A number of strategies for imparting water solubility to C₆₀ are known, e.g., the introduction of water-solubilizing substituents such as carboxylic acid^{11,12)} or polyethylene glycol groups^{13,14)} and the formation of supramolecular complexes with water-soluble

host molecule(s) such as cyclodextrin(s).^{15,16)} However, water-soluble fullerenes prepared by the above methods often form aggregates or precipitates, which decreases the efficiency of generating activated oxygen in aqueous solution.

In recent years, drug delivery systems utilizing enhanced permeation and retention effects, such as those based on macromolecules and micelles, have attracted much attention due to being able to suppress side effects induced by drug dispersion.^{17–19)} However, despite the above progress, the aggregation of fullerene derivatives in water and their biomedical applica-

Chart 1.

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Fig. 1. UV-Vis Spectrum of **WSF** in H₂O (7.83 μM)

tions have been insufficiently investigated.

Herein, we synthesized a novel water-soluble fulleropyrrolidine bearing an *N*-polyethylene glycol (PEG) pyridinium unit (denoted as **WSF**) and correlated its water solubility with ultrasonication time and aggregate size, additionally examining photodynamic activity.

Results and Discussion

The synthesis of **WSF** is shown in Chart 1. PEGylation reagent **1** was synthesized from tetraethylene glycol monomethyl ether **2** and *p*-nitrobenzenesulfonyl chloride **3** in dry tetrahydrofuran (THF) in the presence of triethylamine, being subsequently reacted with fulleropyrrolidine **4**²⁰ in chlorobenzene at 85°C to afford precursor **5** in moderate yield. Finally, anion-exchange of **5** on Dowex 1X8 Cl-form furnished **WSF** in quantitative yield.

The molar absorption coefficient of **WSF** in ethanol (at 254 nm) was determined as 102000 L mol⁻¹ cm⁻¹. Next, **WSF** was dispersed in water upon ultrasonication (Branson B-1200, 60 W, 47 kHz) performed at room temperature to 40°C, and the obtained dispersion was shaken at 200 rpm for 1 h at 25°C and centrifuged at 10000 rpm for 1 h and the supernatant was obtained. After the above operation was repeated three times, the final supernatant was characterized by UV-Vis spectrometry (Fig. 1). Specifically, the supernatant (4 mL) was freeze-dried, and the residue was dissolved in ethanol, with the concentration of **WSF** in water calculated based on the absorption of the ethanolic solution.

As a result, the solubility of **WSF** in water was correlated with ultrasonication time, with saturation (60–80 μM) observed after 12 h (Fig. 2).

After solubility determination, we evaluated the particle size of **WSF** aggregates by dynamic light scattering (DLS) analysis using the Marquardt method. **WSF** was confirmed to form aggregates in water, with the average particle size under sonication-free conditions equaling *ca.* 338 nm and decreasing with increasing sonication time. Despite the same sonication time, solutions of different concentrations were obtained, which confirmed the dependence of particle size on the concentration of **WSF** (Fig. 3). Additionally, we investigated the temporal stability of **WSF** aggregates prepared by 12-h ultrasonication, with the almost unchanged particle size implying aggregate stability (Fig. 4).

Fig. 2. Correlation between Ultrasonication Time and **WSF** Concentration Fig. 3. Correlation between **WSF** Concentration and Particle Size of Aggregates

Next, phosphate buffer (10 mM, pH 7.4) was added to an appropriate amount of **WSF**, sonicated for 15 min × times (ULTRASONIC CLEANER MODEL US-100, 50 kHz, 100 W, total 75 min), centrifuged for 1 h (9100 × *g*, 25°C), and the supernatant was collected to prepare a **WSF** buffer solution. The concentration was determined to be 4 μM by absorbance and molar extinction coefficient. On the other hand, the particle size was not reproducible in DLS measurement, unfortunately. It was guessed that the aggregate size changes in a short time because of the solvent polarity enhancement by the buffer solution.

The photodynamic activity of **WSF** was evaluated using A549 cells, which were seeded in 96-well culture plates and incubated for 24 h in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. **WSF** solutions of various concentrations were added to each well, and the cells were cultured for 24 h, washed twice with phosphate buffered saline, irradiated with a Xenon lamp (35 mW/cm², 400–700 nm) for 0.5 h, and incubated for further 24 h. Subsequently, cell viability was determined using the WST-1 assay, with the obtained results revealing that the toxicity of **WSF** increased with its concentration. The cytotoxicity of **WSF** solution in the dark was observed above about 20 μM, which may be due

Fig. 4. Time Course of Particle Size Distribution of WSF (50.8 μM) in Water after (a) Preparation, (b) 7 d, and (c) 14 d

to its higher interaction with cell membranes because WSF has a cationic moiety in a molecule. However, WSF solutions below 20 μM showed insignificant effects on the cell viability in the dark, whereas exhibited the markedly higher cytotoxicity by the photoirradiation indicating a high photodynamic activity at lower concentrations (Fig. 5). As a result of tetrasodium α,α' -(anthracene-9,10-diyl)bis(methylmalonate) (ABMM) bleaching method,^{21,22)} it was confirmed that singlet oxygen of reactive oxygen species (ROS) was generated from WSF by light irradiation, and so that the photodynamic activity of WSF was considered to be caused by ROS.

In conclusion, WSF was shown to form aggregates with concentration-dependent particle size in aqueous medium, exhibiting photodynamic activity at lower concentrations

Fig. 5. Dose-Dependent Photodynamic Activity of WSF for A549 Cells
Each point represents the mean ± S.E. of 6 experiments.

and thus being a promising photosensitizer for photodynamic therapy.

Experimental

General Methods and Materials ^1H -NMR spectra were recorded on a JEOL ECS-400 instrument. Chemical shifts are reported in parts per million (ppm) relative to a tetramethylsilane internal standard (0.0 ppm). ^1H -NMR data are reported as follows: Chemical shift (δ ppm), integration, multiplicity, and coupling constant (J , Hz). ^{13}C -NMR data are reported as follows: chemical shift (δ ppm). Multiplicities are reported using the following abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; and br, broad. Only the strongest and/or structurally relevant IR peaks are reported (cm^{-1}) on JASCO FT/IR-7000. All mass spectra were measured on a Bruker Autoflex II matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometer. Column chromatography was performed using silica gel (BW-300 Fuji Silysia Chemical, Ltd., Japan). Analytical thin layer chromatography was performed on aluminum plates pre-coated with silica gel (Merck, TLC Silica gel 60F₂₅₄). Dynamic light scattering was measured by Otsuka Electronics Co., Ltd. (Japan) DLS-8000HL. UV-Vis spectra were recorded on Hitachi U-2000.

Synthetic Procedures

3-(*N*-Methylfullero[1,2-*c*]pyrrolidin-2-yl)-1-(3,6,9,12-tetraoxatridecyl)pyridin-1-ium Chloride (WSF)

Under N_2 atmosphere, a solution of fulleropyrrolidine derivative **4**¹⁰⁾ (299 mg, 0.35 mmol) in dry chlorobenzene (5 mL) was treated with a solution of pegylation reagent **1** (235 mg, 0.60 mmol) in the same solvent (10 mL) and stirred at 85°C for 24 h. The reaction mixture was evaporated and taken up in diethyl ether, and the residue was purified by column chromatography. **4** (6 mg) was recovered from the chloroform–ethyl acetate (5:1) eluate, whereas precursor **5** (372 mg, 85%) was obtained from the chloroform–methanol (5:1) eluate and reprecipitated in chloroform–diethyl ether.

Anion exchange resin (50 g) was sequentially washed with water (3 × 250 mL), 1 wt% aqueous HCl (250 mL), ion-exchanged water (3 × 250 mL), methanol (250 mL), and ion-exchanged water (3 × 250 mL). After the resin was packed in the column, as solution of **5** (187 mg, 0.15 mmol) in methanol

(75 mL) was applied, affording **WSF** (162 mg, quant.) as a dark brown solid. Mp >300°C. IR (KBr): 2868 (CH), 1108 (C–O–C), 526 cm⁻¹ (C60). ¹H-NMR (400 MHz, CDCl₃) δ: 2.87 (3H, s, N–CH₃), 3.33 (3H, s, CH₃–O), 3.43–3.69 (12H, m, CH₂ in ethylene glycol unit), 3.91–4.13 (2H, m, β-CH₂), 4.42, 5.03 (each 1H, d, J=9.6 Hz, 5-H), 5.36 (3H, brs, 2-H, α-CH₂), 8.13 (1H, dd, J=6.5, 7.5 Hz, 5'-H), 8.92 (1H, brs, 4'-H), 9.81 (2H, brs, 2',6'-H). ¹³C-NMR (100.5 MHz, CDCl₃) δ: 39.95 (N–CH₃), 59.04 (CH₃–O), 68.91 (3 or 4-C), 69.63, 69.86, 70.11, 70.39, 70.48, 70.63, 71.86, (CH₂ in ethylene glycol unit), 76.23, (3 or 4-C), 127.78, 135.71, 135.74, 136.79, 137.97, 138.96, 139.68, 140.29, 140.39, 140.51, 141.52, 141.69, 141.78, 141.88, 142.04, 142.07, 142.10, 142.29, 142.58, 142.74, 142.78, 142.87, 143.17, 143.24, 144.28, 144.50, 144.54, 144.81, 145.12, 145.19, 145.22, 145.28, 145.46, 145.48, 145.61, 145.63, 145.80, 146.02, 146.09, 146.21, 146.26, 146.32, 146.35, 146.46, 146.52, 147.36, 147.49, 151.01, 152.72, 155.40 (C₆₀). MALDI-TOF-MS (dithranol) *m/z* 1045 ([M–Cl]⁺).

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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