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Relation	


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Water-Solubilization of Fullerene Derivatives by β-(1,3-1,6)-D-Glucan and Their Photodynamic Activities toward Macrophages

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Abstract: Anionic and neutral fullerene derivatives were dissolved in water by β-(1,3-1,6)-D-glucan (β-1,3-glucan) as a solubilizing agent. In the water-solubilized complexes, the concentrations of fullerene derivatives were *ca*. 0.30 mM and the average particle sizes were *ca*. 90 nm. The β-1,3-glucan complexed fullerene derivative with a carboxylic acid was found to have higher photodynamic activity toward macrophages under visible-light irradiation (λ > 610 nm) when compared with that of other β-1,3-glucan-complexed fullerene derivatives. This result suggests that carboxylic acid moieties in the complex enhance the binding affinity with β-1,3-glucan-receptors on the surface of macrophages when β-1,3-glucan is recognized. In contrast, all β-1,3-glucan complexed fullerene derivatives showed no photodynamic activity toward HeLa cells under the same conditions.

Introduction

The backbone of β-1,3-glucans consist of glucose residues linked by β-1,3-glycosidic bonds and often β-1,3-glucans have glucose side chain residues linked by β-1,6-glycosidic bonds.[1] These β-1,3-glucans are recognized by immune cell-specific β-1,3-glucan receptors such as dectin-1 and complement receptor 3, which are expressed on the cell surface of murine and human macrophages.[2] Thus, β-1,3-glucans represent a very good candidate for the development of novel biomaterials that target macrophages.[3] Furthermore, β-1,3-glucan can form inclusion complexes with hydrophobic molecules, in a similar manner to starch and amylose. For example, schizophillan, which is a member of the β-1,3-glucan family, is known to form inclusion complexes with carbon nanotubes,[4] polyaniline,[5] polythiophene[6] and porphyrins.[7] Therefore, β-1,3-glucan is expected to act as a drug carrier in treating macrophage-associated diseases.

Fullerenes and their derivatives are photosensitizers that are highly efficient in generating light-induced reactive oxygen species (ROS) and function as efficient DNA cleavage agents,[8] protein cleavage agents[9] and potential sensitizers in photodynamic therapy (PDT).[10,11] These applications in biology and medicine have motivated efforts to produce water-solubilized fullerenes using solubilizing agents.[12] In particular, γ-cyclodextrin-complexed C_{60} derivatives have higher photodynamic activities than γ-cyclodextrin-complexed C_{60} and C_{70}.[13] In this paper, β-1,3-glucan was used as a water-solubilizing agent for fullerene derivatives and as a drug-targeting carrier for macrophages. Photodynamic activities of the β-1,3-glucan-complexed C_{60} derivatives toward macrophages were assayed under visible-light irradiation (λ > 610 nm). It is envisaged that the β-1,3-glucan-complexed C_{60} derivatives will show higher levels of intracellular uptake than the cyclodextrin-complexed C_{60} derivatives because of the formation of multi-point interactions between the substituents of the C_{60} derivatives and the cell

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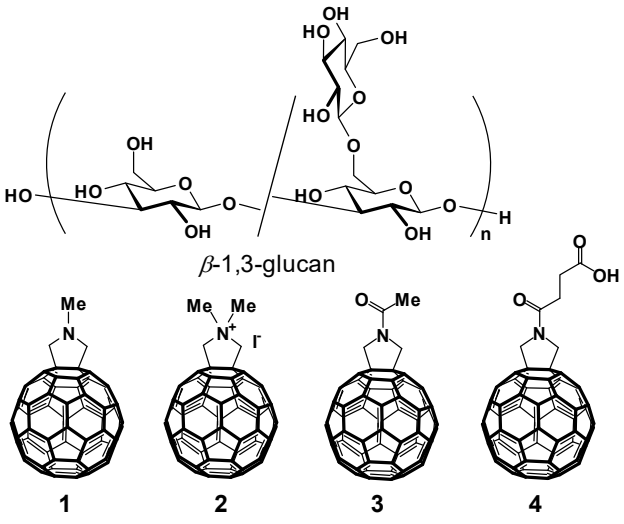

Figure 1. Chemical structures of β-1,3-glucan and compounds **1**–**4**.

surfaces.

Results and Discussion

Preparation of β-1,3-glucan-complexed fullerene derivatives

We used β-glucan purified from black yeast (*Aureobasidium pullulans*). The β-1,3-glucan-complexed C_{60} derivatives **1**–**4** (Figure 1) were prepared using a mechanochemical high-speed vibration milling (HSVM) apparatus (Schemes S1 and S2).[14] The mixtures were dissolved in water and a brown emulsion was sonicated using an ultrasonic bath (180 W, 42 kHz, 5510 Branson Ultrasonic Corp.) for 2 h. Samples were then centrifuged for 20 min at 4500 × *g* to remove the non-disperse C_{60} derivative.

Figure 2 shows UV–vis absorption spectra of the β-1,3-glucan-complexed C_{60} derivatives in water. The spectra reveal that **1**, **2**, **3** and **4** were dissolved in water by β-1,3-glucan as a solubilizing agent (Figure 2, black, green, blue and red lines). The solutions for **1**, **3** and **4** were stable for at least one month at room temperature. In contrast, the β-1,3-glucan-complexed **2** was not appreciably soluble in water (Figure 2, green line). Kano et al. reported that the binding constants of cyclodextrin for anionic porphyrin derivatives were much larger than that of a cationic porphyrin derivative.[15] This difference can be attributed to electrostatic repulsion within the positively polarized interior of the cyclodextrin cavity, resulting from the inductive effect of the ethereal oxygen atoms. Given that cyclodextrin, which consists of glucose residues, has a similar cavity to that of β-1,3-glucan, it is perhaps not surprising that we were unable to form β-1,3-glucan-complexed **2**. To evaluate the concentrations of **1**, **3** and **4** in the solution of the β-1,3-glucan-complexed C_{60} derivatives, these complexes were decomposed by heating under reflux for 24 h in the presence of H_2SO_4 (1.0 M), which cleaves β-1,3-glucan and results in the precipitation of **1**, **3** and **4**. The precipitated materials were then dissolved in 1,2-dichlorobenzene; however, a fraction of the precipitates of **1**, **3** and **4** remained insoluble. The result is similar to that reported previously in which C_{60} in a solid or solution of water-soluble calix[8]arene derivative•C_{60} complex cannot be extracted in carbon disulfide or toluene.[16] Because it is very difficult to determine directly the concentrations of **1**, **3** and **4**, molecular extinction coefficients of the β-1,3-glucan-complexed **1**, **3** and **4** in water were substituted by those of lipid membrane-incorporated **1** prepared via fullerene exchange from the γ-cyclodextrin cavity to liposomes by heating at 80 °C (ε_{330} = 4.96 × 10^4 dm^3 mol^{-1} cm^{-1}) (Figure S1). The concentrations of **1**, **3** and **4** were thus determined to be 0.30, 0.32 and 0.38 mM, respectively, in 2 g L^{-1} β-1,3-glucan solutions (Table S1).

Morphology and characteristics of β-1,3-glucan-complexed fullerene derivatives

The morphologies of the β-1,3-glucan-complexed **1**, **3** and **4** were observed by transmission electron microscopy (TEM; Figure 3). In all TEM micrographs, short rod-like structures (globular structures) with lengths of approximately 50–100 nm and diameters of about 20 nm were abundantly observed (Figures 3b–d and 3f–h). These globular structures are wider than the β-1,3-glucan in the absence of C_{60} derivatives (Figures 3a and 3e). The formation of the globular structures is often observed[17] and is similar to those of the β-1,3-glucan-complexed C_{70}.[18]

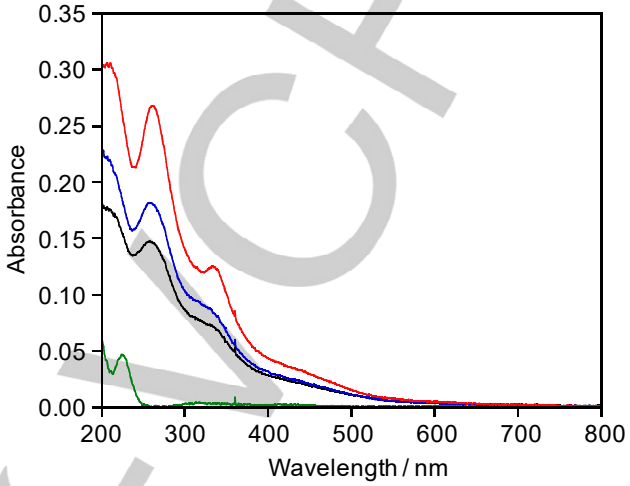

Figure 2. UV-vis absorption spectra of the β-1,3-glucan-complexed **1** (black line), **2** (green line), **3** (blue line) and **4** (red line) in water (1 mm cuvette, 25 °C).

Dynamic light scattering (DLS) measurements gave further information about sizes of the β-1,3-glucan-complexed **1**, **3** and **4** (Table 1). The average hydrodynamic diameters (D_{hy}) were estimated to be ~90 nm in all complexes. These average D_{hy} are slightly larger than those observed by TEM (Figure 3) in the β-1,3-glucan-complexed **1**, **3** and **4** with folded structures because the mean hydrodynamic radii are calculated using the standard Stokes−Einstein equation for a spherical particle.[19]

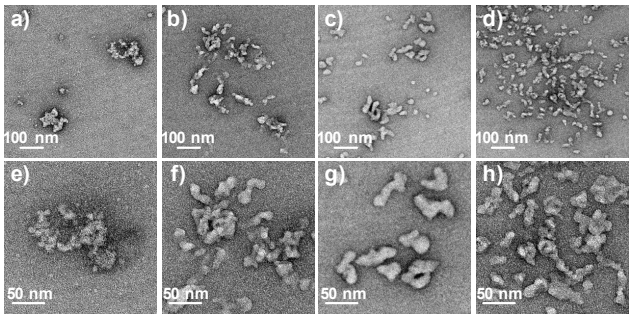

Figure 3. TEM images of a) and e) β-1,3-glucan only, the β-1,3-glucan-complexed b) and f) **1**, c) and g) **3**, and d) and h) **4** after HSVM treatment.

We characterized surface potentials of the β-1,3-glucan-complexed **1**, **3** and **4** using zeta potential measurements. As shown in Table 1, the detected surface charges of these complexes showed small differences regardless of the fullerene derivative. This observation suggests that the carboxylic acid of **4**

does not dissociate into carboxylate at pH values around neutrality.

Table 1. Average particle sizes (nm) and zeta potentials of *β*-1,3-glucan-complexed **1**, **3** and **4**.

Complex	Average particle size [nm] [a]	PDI [a,b]	Zeta potential [mV] [a]
β-1,3-glucan-complexed **1**	84.8	0.103	-20 ± 7
β-1,3-glucan-complexed **3**	92.2	0.082	-13 ± 3
β-1,3-glucan-complexed **4**	90.5	0.170	-20 ± 1

[a] Each experiment was carried out three times. [b] PDI means the polydispersity index.

Photodynamic activity for macrophage and HeLa cells

The photodynamic activities of the *β*-1,3-glucan-complexed **1**, **3** and **4** toward macrophages (mouse macrophage cell line RAW264.7) were subsequently evaluated. After incubation with 0.5–15.0 μM fullerene derivatives of these complexes for 24 h, the cells were exposed to light with a wavelength ≥ 610 nm (610–720 nm). The results are shown in Figure 4. For the *β*-1,3-glucan-complexed **1** and **3** treated cells, no cytotoxicities were observed with or without light exposure (Figures 4a and S2a). Conversely, the photodynamic activity of the *β*-1,3-glucan-complexed **4** was drug dose-dependent and the half maximal inhibitory concentration (IC_{50}) value was estimated to be fullerene concentrations of *ca*. 5.3 μM in combination with light irradiation (Figure 4a red line). In contrast, all of *β*-1,3-glucan complexes exhibited no photodynamic activities against HeLa cells (Figures 4b and S2b). Thus, the *β*-1,3-glucan-complexed **4** clearly showed much higher photodynamic activity toward RAW264.7 cells when compared with that of HeLa cells.

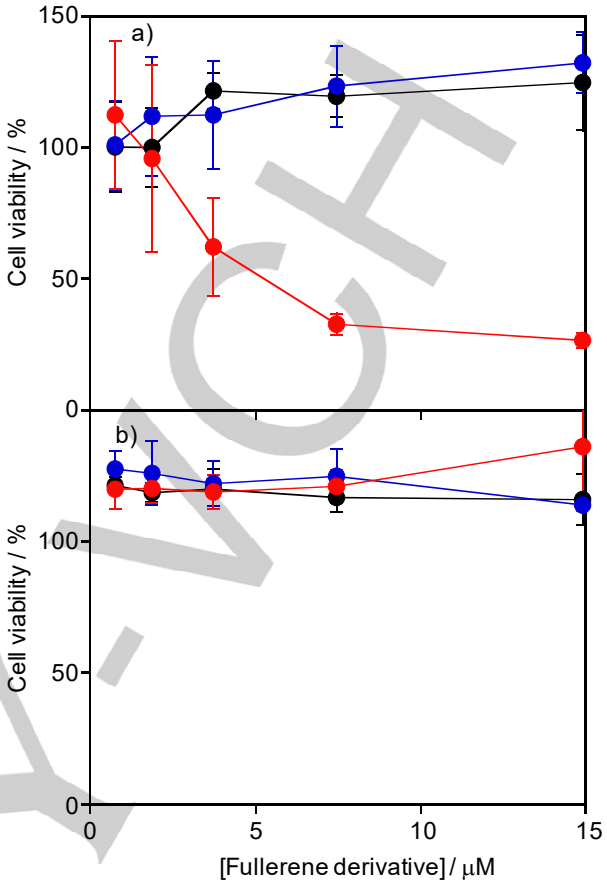

Figure 4. Concentration dependence of the cytotoxicity of the *β*-1,3-glucan-complexed **1** (black line), **3** (blue line) and **4** (red line) for a) RAW cells and b) HeLa cells under light irradiation (610–720 nm, 30 min).

Singlet oxygen generation abilities of *β*-1,3-glucan-complexed fullerene derivatives

The amount of singlet oxygen molecules ($^1O_2$) generated was measured according to a chemical method using the 9,10-anthracenedipropionic acid disodium salt (ADPA)[20] as a detector to determine the differences in biological activities of the *β*-1,3-glucan-complexed **1**, **3** and **4** compounds. The level of ADPA absorption at 400 nm (absorption maximum for ADPA) was monitored as a function of time following the irradiation of 15 μM samples of the complexes (Figure 5). The results indicated that the different *β*-1,3-glucan complexes generated $^1O_2$ in the order of **4** ≈ **1** > **3**. This order does not correlate with the photodynamic activities of *β*-1,3-glucan-complexed **1**, **3** and **4** toward the macrophages, indicating that the amount of $^1O_2$ generated is not the sole factor that defines activity of these complexes.

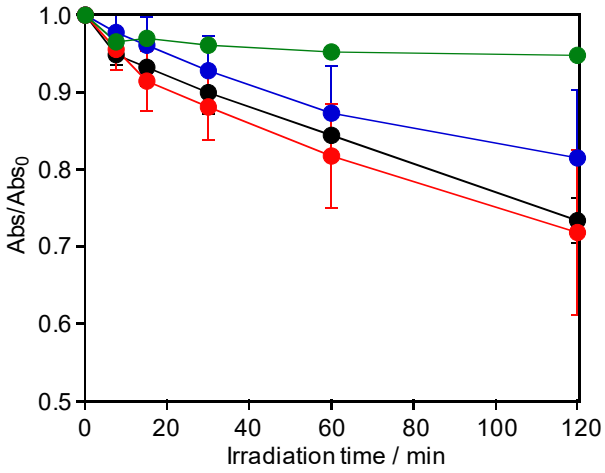

Figure 5. Time-dependent bleaching of 9,10-anthracenedipropionic acid disodium salt (ADPA) caused by singlet oxygen generated from *β*-1,3-glucan-complexed **1** (black line), **3** (blue line) and **4** (red line), and without a *β*-1,3-glucan-complexed fullerene derivative (green line). Changes in the ADPA absorption at 400 nm upon photoirradiation (> 610 nm, 15 mW cm^{-2}) were monitored as a function of time (Abs_0: initial absorbance). [**1**, **3** or **4**] = 15 μM, [ADPA] = 25 μM: under an oxygen atmosphere at 25 °C.

Intracellular uptake of *β*-1,3-glucan-complexed fullerene derivatives

The uptake of the *β*-1,3-glucan-complexed **1**, **3** and **4** was determined by FACS. Macrophage cells were incubated for 24 h with a mixture of these complexes ([**1**, **3** or **4**] = 4 μM). As shown in Figure S3, the *β*-1,3-glucan-complexed **1**, **3** and **4** possessed similar intensities in the range of 570–600 nm when they were excited at the wavelength (λ_{ex} = 488 nm) used in FACS. The cellular uptake of the *β*-1,3-glucan-complexed **4** by RAW264.7 cells was greater than those of the *β*-1,3-glucan-complexed **1** and **3** (Figure 6a). Furthermore, no uptake of *β*-1,3-glucan complexes by HeLa cells were monitored (Figure 6b). These results are consistent with the selective photodynamic activity of *β*-1,3-glucan-complexed **4** toward RAW264.7 cells. *β*-Glucan•DNA complexes and *β*-glucan derivatives, such as glucan phosphate and carboxymethyl-pachyman, bind to *β*-1,3-glucan receptors including the dectin-1 more strongly than native *β*-glucan.[21] That is, recognition of *β*-1,3-glucan receptors for *β*-1,3-linked and *β*-1,6-linked glucans is expected to be reinforced by the presence of acid moieties such as phosphoric acid and carboxylic acid. Consequently, the high cellular uptake of the *β*-1,3-glucan-complexed **4** is expected because of the carboxylic acid moiety of **4**. Notably, Broun et al. reported that glucan phosphate and carboxymethyl-patchman exhibit similar binding properties toward Dectin-1 Fc chimera,[20a] suggesting that the carboxylic acid moieties does not need to dissociate. This result is therefore consistent with the results observed in the current study for the surface potentials of the *β*-1,3-glucan-complexed **4**, which were determined using zeta potential measurements (Table 1). However, it has been reported that the expression level of endogenous dectin-1 is low in RAW264.7 cells.[22] Therefore, high binding affinity of *β*-1,3-glucan-complexed **4** to its receptors may notably affect uptake efficiency by RAW264.7 cells, resulting in selective photodynamic activity. This explanation is supported by the observation of no uptake of *β*-1,3-glucan-complexed **4** by HeLa cells, which do not express dectine-1 (Figure 6b).

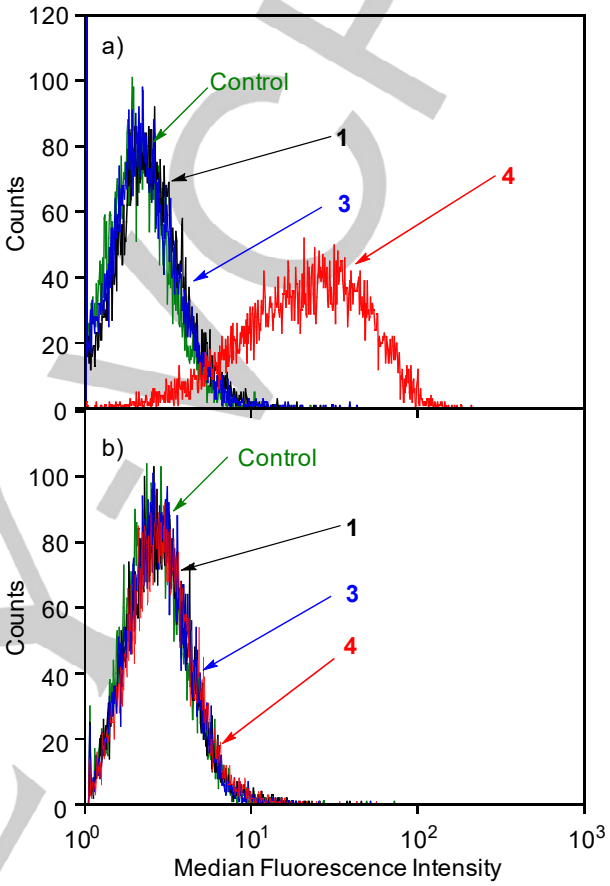

Figure 6. Flow cytometry analysis for the detection of **1**, **3** and **4** in a) RAW cells and b) HeLa cells; untreated cells (green line), cells treated with the *β*-1,3-glucan-complexed **1** (black line), **3** (blue line) and **4** (red dotted line) ([C_{60}-**1**, **3** or **4**] = 4 μM).

Conclusions

In this study, *β*-1,3-glucan solubilized C_{60} derivatives **1**, **3** and **4** in water, and the solutions of the complexes were stably stored for at least one month. Among them, only *β*-1,3-glucan-complexed **4** showed a high level of photodynamic activity toward RAW264.7 cells under photoirradiation at long wavelengths (610–740 nm). The high level of photodynamic activity was strongly dependent on cellular uptake by RAW264.7 cells. No cytotoxicities were observed when the *β*-1,3-glucan-complexed **1** and **3** were added to RAW cells and all *β*-1,3-glucan complexes added to HeLa cells under light exposure displayed very low cellular uptake. The selectivity of the *β*-1,3-glucan-complexed fullerene derivative is expected to function as a drug carrier in macrophage-associated disease therapy, and represents a future continuation of this line of study.

Experimental Section

Materials

β-(1,3-1,6)-D-glucan was supplied by Daiso Co. Ltd., (Hyogo, Japan) and was further purified in-house. Compounds **1**,[23] **2**,[24] **3**[25] and **4**[12f] were synthesized in accordance with literature procedures.

Preparation of the *β*-1,3-glucan-complexed fullerene derivatives

1, **2**, **3** or **4** (2.00×10^{-6} mol) and *β*-1,3-glucan (10.0 mg) were placed in an agate capsule with two agate-mixing balls. The mixture was mixed vigorously at 30 Hz for 20 min using a high-speed vibration mill (MM 200; Retsch Co., Ltd., Haan, Germany). The solid mixture was suspended in saline (5.0 mL) to produce a brown emulsion. The resulting suspension was sonicated using an ultrasonic bath (180 W, 42 kHz, 5510 Branson Ultrasonic Corp., Connecticut, USA) for 2 h. After centrifugation (4500 × *g*, 25 °C, 20 min), the non-dispersed **1**, **2**, **3** or **4** was removed from the solution. The concentration of **1**, **3** or **4** in the complex with *β*-1,3-glucan, determined by the molar absorption coefficient for lipid membrane-incorporated C_{60} derivative **1** ($\varepsilon_{330} = 4.96 \times 10^4$ dm^3 mol^{-1} cm^{-1}) in place of those for the *β*-1,3-glucan-complexed **1**, **3** or **4**.

UV–vis absorption spectra

UV–vis spectra were recorded using a UV-2550PC spectrophotometer (Shimadzu Corp., Kyoto, Japan). All experiments were performed at 25 °C and a 1 mm cuvette was used.

Dynamic light scattering (DLS) analysis

The hydrodynamic diameters of *β*-1,3-glucan-complexed **1**, **3** or **4** were measured by a Zetasizer (Nano ZS, Malvern Instruments Ltd., Malvern, UK). The instrument consisted of a He/Ne laser operating at 633 nm and 10 mW. The DTS Nano version 5.00 software was used and is supplied by the manufacturer (Malvern Instruments Ltd., Malvern, UK).

Zeta-potential measurements

The zeta potentials of the *β*-1,3-glucan-complexed **1**, **3** or **4** were measured on an instrument for electrophoretic light scattering with a laser Doppler system (Nano ZS, Malvern Instruments Ltd., Malvern, UK).

Transmission electron microscopy (TEM)

The *β*-1,3-glucan-complexed **1**, **3** or **4** were assessed by transmission electron microscopy using the negative staining method by phosphotungstate. A solution of the complexes was cast on an ultrathin carbon-deposited Cu grid (Cu200, JEOL Datum Ltd., Tokyo, Japan) and dried in a desiccator overnight followed by in vacuo for 1 h. TEM observations were carried out by a JEM-2200FS field emission electron microscope (JEOL Ltd., Tokyo, Japan) with an acceleration voltage of 200 kV.

Photodynamic activity experiments

RAW264.7 or HeLa cells were maintained in CO_2 Independent Medium (Gibco BRL, Eggenstein, Germany) supplemented with 10% fetal calf serum at 37 °C in 5% CO_2. For experiments conducted to determine the photodynamic activities of the *β*-1,3-glucan-complexed **1**, **3** or **4**, the cells were seeded in 48-well culture plates at a density of 1.7×10^4 cells per well. After growing cells overnight, the cells were incubated with the *β*-1,3-glucan-complexed **1**, **3** or **4** for 24 h in the dark. The cells were washed with PBS and exposed to light for 30 min at 25 °C. Light irradiation was performed 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 of 610 nm. The power of the light at the cellular level was 9 mW cm^{-2} (610–740 nm). To measure the viability of cells as a percentage ratio relative to the cells that were not treated, a WST-8 assay was conducted 24 h after the photoirradiation process using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan), according to the manufacturer's instructions.

Singlet oxygen detection by a chemical method

$^1O_2$ generation was confirmed by a chemical method using 9,10-anthracenedipropionic acid disodium salt (ADPA)[19] as the detector. For the ADPA bleaching method, all samples were prepared in a deuterium oxide solution. The concentration of the *β*-1,3-glucan-complexed **1**, **3** or **4** and ADPA in the mixed solutions was [**1**, **3** or **4**] = 15 μM and [ADPA] = 25 μM. Before photoirradiation, all samples were bubbled with oxygen for 30 min to generate aerobic conditions. The 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 610 nm. The filter was cooled by passing it through a water filter. The power of the light was 15 mW cm^{-2} (over 610 nm) at the sample level.

Flow cytometry analysis

RAW264.7 or HeLa cells were incubated with the *β*-1,3-glucan-complexed **1**, **3** or **4** at a concentration of 4 μM for 24 h at 37 °C in 5% CO_2. Following incubation, the cells were washed with phosphate-buffered saline (PBS), detached with a 0.05% trypsin/0.02% EDTA-PBS (EDTA = ethylenediaminetetraacetic acid) solution and subsequently suspended in PBS. The suspended cells were added directly to a FACSCalibur flow cytometer (Becton–Dickinson, Franklin Lakes, NJ, USA). Analyses were gated to include single cells on the basis of forward and side light scattering and were based on the acquisition of data from 10000 cells. Log fluorescence values were determined and displayed as single-parameter histograms. The geometric mean fluorescence intensity was calculated by the CellQuest 3.0 program (Becton–Dickinson, Franklin Lakes, NJ, USA).

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Keywords: fullerenes • host-guest chem. • macrophage • medicinal chem. • polysaccharides

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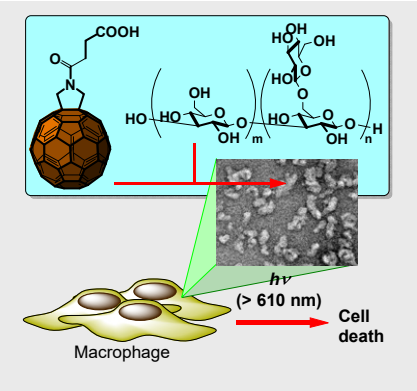
A *β*-1,3-glucan complexed fullerene derivative with a carboxylic acid has higher photodynamic activities toward macrophages under visible-light irradiation (λ > 610 nm) when compared with that of *β*-1,3-glucan-complexed with other fullerene derivatives.



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Water-Solubilization of Fullerene Derivatives by *β*-(1,3-1,6)-D-Glucan and Their Photodynamic Activities toward Macrophages