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Title	Formation of $\beta$ - (1,3 - 1,6) - D - glucan - complexed [70]fullerene and its photodynamic activity towards macrophages
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Relation	

## Formation of $\beta$ -(1,3-1,6)-D-glucan-complexed [70]fullerene and its photodynamic activity towards macrophages†

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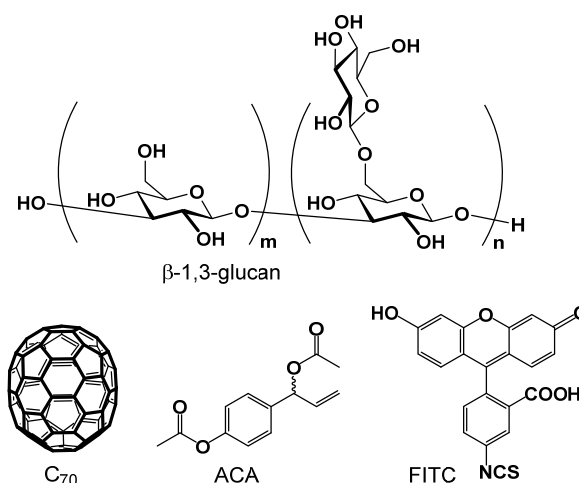
[70]Fullerene was dissolved in water by the complexation with  $\beta$ -1,3-glucan using a mechanochemical high-speed vibration milling apparatus. The photodynamic activity of  $\beta$ -1,3-glucan-complexed  $C_{70}$  was highly dependent on the expression level of dectin-1 on the cell surfaces of macrophages. The photodynamic activity increased as a result of a synergistic effect between  $\beta$ -1,3-glucan-complexed 1'-acetoxychavicol acetate and the  $C_{70}$  complex.

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### Introduction

$\beta$ -(1,3-1,6)-D-Glucans ( $\beta$ -1,3-glucan) consist of only glucose residues and a backbone linked by  $\beta$ -1,3-glycosidic bonds with sidechains joined by  $\beta$ -1,6 linkages.<sup>1</sup>  $\beta$ -1,3-Glucan itself has been described to have low toxicity, excellent biocompatibility and several important medicinal effects, such as antitumor, immunomodulatory, antimicrobial, antinociceptive, anti-inflammatory, prebiotic, antioxidant, and antidiabetic activities.<sup>2</sup> As  $\beta$ -1,3-glucan can form inclusion complexes with hydrophobic guest molecules, such as carbon nanotubes,<sup>3</sup> polyaniline,<sup>4</sup> polythiophen<sup>5</sup> and porphyrins,<sup>6</sup> these medicinal effects could be improved by the synergistic effect of  $\beta$ -1,3-glucan and the hydrophobic molecule forming the complexes. Furthermore,  $\beta$ -1,3-glucan has been recognised to be immune to cell-specific  $\beta$ -1,3-glucan receptors such as dectin-1 and complement receptor 3 expressed on the surface of murine and human macrophages.<sup>7,8</sup> That is,  $\beta$ -1,3-glucan acts as not only a solubilising agent but also as a drug carrier. Therefore, attention has been paid to the solubilisation of drugs in water using biocompatible natural products, such as liposomes,<sup>9</sup> cyclodextrins<sup>10</sup> and peptides,<sup>11</sup> as solubilising agents. We have previously reported that 1'-acetoxychavicol acetate (ACA), an inhibitor of the activation of nuclear factor  $\kappa$ B (NF- $\kappa$ B) regulating the expression of proinflammatory cytokines, chemokines, and growth factors,<sup>12</sup> can be solubilised in aqueous solution by

complexing with  $\beta$ -1,3-glucan. The obtained complexes showed the anti-inflammatory effect on contact dermatitis.<sup>13</sup> Therefore,  $\beta$ -1,3-glucan can be expected to act as a drug carrier in the therapy of macrophage-associated diseases, such as rheumatoid arthritis. Because photodynamic therapies have been used in the treatment of not only cancers but also rheumatoid arthritis,<sup>14</sup> it is very important to develop a drug carrier that can transfer the photosensitisers used in photodynamic therapies into macrophages. We selected [70]fullerene ( $C_{70}$ ) as a hydrophobic guest molecule, as it is known to be a photosensitiser with highly efficient light-induced generation of reactive oxygen species (ROS).<sup>15–17</sup> In liposomes, the photoactivity of  $C_{70}$  was much higher than that of  $C_{60}$  under photoirradiation in the range of 400–700 nm. In this paper, we report (i) the solubilisation of  $C_{70}$  in water by the formation of a complex with  $\beta$ -1,3-glucan isolated from *Aureobasidium pullulans* GM-NH-1A1 strain (black yeast, a mutant of the strain K-1),<sup>18,19</sup> (ii) the comparison of the photodynamic activities of  $\beta$ -1,3-glucan-complexed  $C_{70}$  toward several cells with a different expression level of dectin-1 on the cell surfaces and (iii) the combined effect of  $\beta$ -1,3-glucan-complexed  $C_{70}$  with  $\beta$ -1,3-glucan-complexed ACA.<sup>13</sup>



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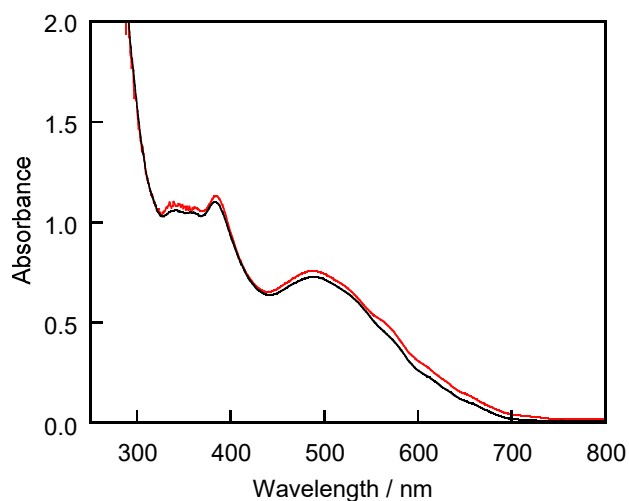
† Electronic Supplementary Information (ESI) available: TEM images. See DOI: 10.1039/x0xx00000x

## Results and discussion

### Preparation of $\beta$ -1,3-D-glucan-complexed $C_{70}$

$\beta$ -1,3-Glucan-complexed  $C_{70}$  and  $\beta$ -1,3-glucan-complexed fluorescein isothiocyanate (FITC) were prepared using a mechanochemical high-speed vibration milling (HSVM) apparatus.<sup>20</sup>  $\beta$ -1,3-Glucan-complexed ACA was prepared according to our previously reported method.<sup>13</sup>

To evaluate the concentration of  $C_{70}$  in the solution of  $\beta$ -1,3-glucan-complexed  $C_{70}$ , the complex was decomposed by heating under reflux for 24 h in the presence of  $H_2SO_4$  (1.0 M) to cleave  $\beta$ -1,3-glucan and then dissolving the resulting precipitated  $C_{70}$  in 1,2-dichlorobenzene. However, a part of the  $C_{70}$  precipitate remained insoluble. This was similar to a previous report in which  $C_{60}$  in a solid or solution of a water-soluble calix[8]arene derivative• $C_{60}$  complex could not be extracted in carbon disulfide or toluene.<sup>21</sup> As it was difficult to directly determine the concentration of  $C_{70}$ , the molecular extinction coefficient of  $\beta$ -1,3-glucan-complexed  $C_{70}$  in water was compared with that of lipid membrane-incorporated  $C_{70}$  prepared using fullerene exchange from the  $\gamma$ -cyclodextrin cavity to liposomes by heating at 30 °C ( $\epsilon_{382} = 4.1 \times 10^4 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$ ).<sup>22</sup> Fig. 1 shows a UV-vis absorption spectra of the lipid-membrane-incorporated  $C_{70}$  and  $\beta$ -1,3-glucan-complexed  $C_{70}$  in water. The absorption spectra revealed that  $C_{70}$  could be dissolved in water by using  $\beta$ -1,3-glucan as a solubilising agent. Consequently, the concentration of  $C_{70}$  was found to be 0.27 mM in 2.7 g  $L^{-1}$   $\beta$ -1,3-glucan solution. The solution for  $C_{70}$  was stable for at least one month at room temperature.



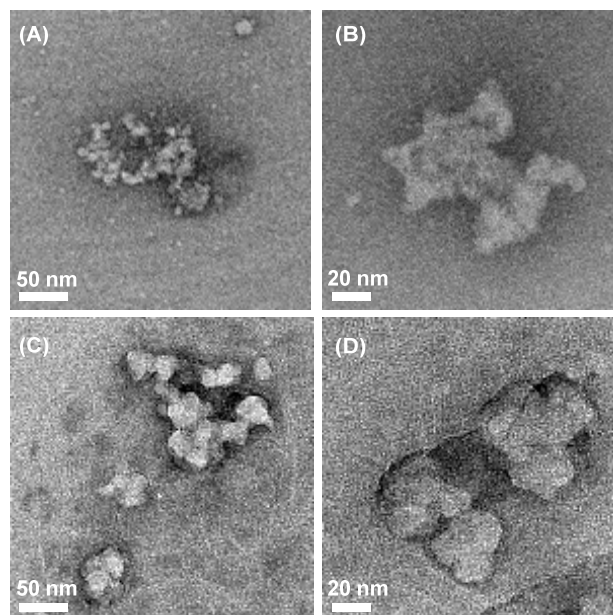
**Fig. 1** UV-vis absorption spectra of the lipid-membrane-incorporated  $C_{70}$  (black line,  $[C_{70}] = 0.25 \text{ mM}$ ,  $[C_{70}]/[\text{lipid}] = 25 \text{ mol}\%$ ) and  $\beta$ -1,3-glucan-complexed  $C_{70}$  (red line,  $[C_{70}] = 0.27 \text{ mM}$ ,  $\beta$ -1,3-glucan:  $2.7 \text{ g L}^{-1}$ ) in water (1 mm cell).

### Morphology and characteristics of $\beta$ -1,3-glucan-complexed $C_{70}$

The morphology of  $\beta$ -1,3-glucan-complexed  $C_{70}$  was observed by transmission electron microscopy (TEM; Figs. 2 and S1). In all the TEM micrographs, the folded structures (globule

structure)<sup>23</sup> with a length of approximately 50–100 nm and a diameter of approximately 20 nm were abundant (Figs. 2C, 2D and S1). The structure was wider than  $\beta$ -1,3-glucan itself (Fig. 2A and 2B). The presence of the folded structures suggests that  $\beta$ -1,3-glucan was shortened owing to the application of high pressure during HSVM and coated the self-aggregates of  $C_{70}$ .

The hydrodynamic diameter ( $D_{hy}$ ) of  $\beta$ -1,3-glucan-complexed  $C_{70}$  was evaluated to be 106 nm (polydispersity index: 0.18) by means of dynamic light scattering (DLS) measurements. This DLS result was consistent with observations made by TEM (Figs. 2C, 2D and S1).



**Fig. 2** TEM images of (A) and (B)  $\beta$ -1,3-glucan only and (C) and (D)  $\beta$ -1,3-glucan-complexed  $C_{70}$  after HSVM treatment.

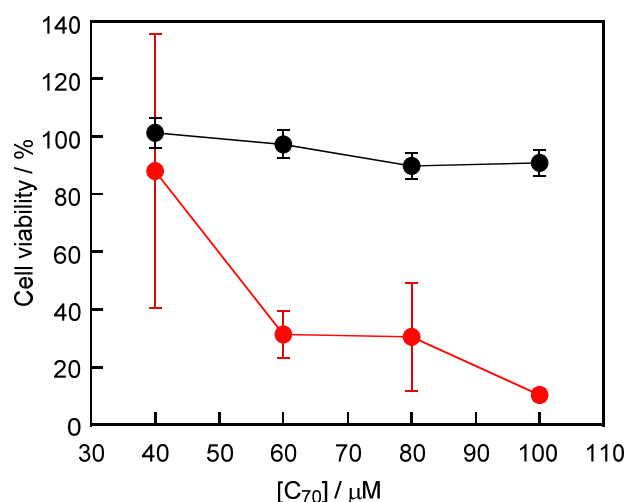
### Photodynamic activity of $\beta$ -1,3-glucan-complexed $C_{70}$ toward RAW264.7

We investigated the photodynamic activities of  $\beta$ -1,3-glucan-complexed  $C_{70}$  toward RAW264.7 cells. After treatment with 40–100  $\mu\text{M}$  of  $C_{70}$ , the cells were photoirradiated in the range of 400–700 nm for 30 min ( $9.1 \text{ mW cm}^{-2}$ ). The viability of the cells 24 h after photoirradiation was measured as a percentage ratio relative to the cells that were not treated with  $C_{70}$  and were not photoirradiated. The results are shown in Fig. 3. No cytotoxicity was observed when  $\beta$ -1,3-glucan-complexed  $C_{70}$  was added to the cells without light exposure (Fig. 3 black line). In contrast, the photodynamic activity of  $\beta$ -1,3-glucan-complexed  $C_{70}$  was drug dose-dependent (Fig. 3 red line) and the half maximal inhibitory concentration ( $IC_{50}$ ) value was estimated to be approximately 50  $\mu\text{M}$  (Fig. 3). The result suggests that  $\beta$ -1,3-glucan-complexed  $C_{70}$  was incorporated by RAW264.7 cells because the photosensitisers remaining in the culture medium were removed before photoirradiation.

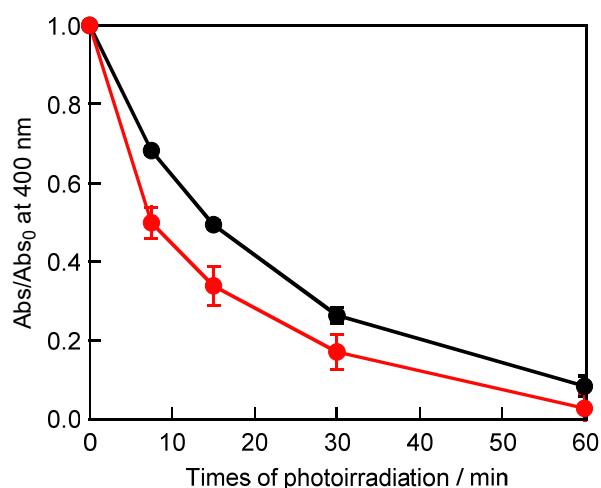
### Singlet oxygen generation ability of $\beta$ -1,3-glucan-complexed $C_{70}$

The amount of singlet oxygen molecules ( $^1O_2$ ) generated was measured according to a chemical method using 9,10-

anthracenediyl-bis(methylene)dimalonic acid (ABDA)<sup>24,25</sup> as a detector to determine the biological activity of  $\beta$ -1,3-glucan-complexed  $C_{70}$  compared with  $\gamma$ -cyclodextrin-complexed  $C_{70}$ . The level of ABDA absorption at 400 nm (absorption maximum for ABDA) was monitored as a function of time following the irradiation at the same concentration of  $C_{70}$  in the complexes ( $[C_{70}] = 15 \mu\text{M}$ ) (Fig. 4). The results indicated that  $\beta$ -1,3-glucan-complexed  $C_{70}$  generated a slightly higher level of  $^1\text{O}_2$  than  $\gamma$ -cyclodextrin-complexed  $C_{70}$ . It was suggested that  $\gamma$ -cyclodextrin-complexed  $C_{70}$  inhibiting energy transfer toward dissolved oxygen in the  $\gamma$ -cyclodextrin cavities to a much greater extent than  $\beta$ -1,3-glucan-complexed  $C_{70}$ .<sup>26</sup> In other words, photoexcited  $C_{70}$  in  $\beta$ -1,3-glucan can readily interact with dissolved oxygen rather than  $\beta$ -1,3-glucan-complexed  $C_{70}$  quenching itself.



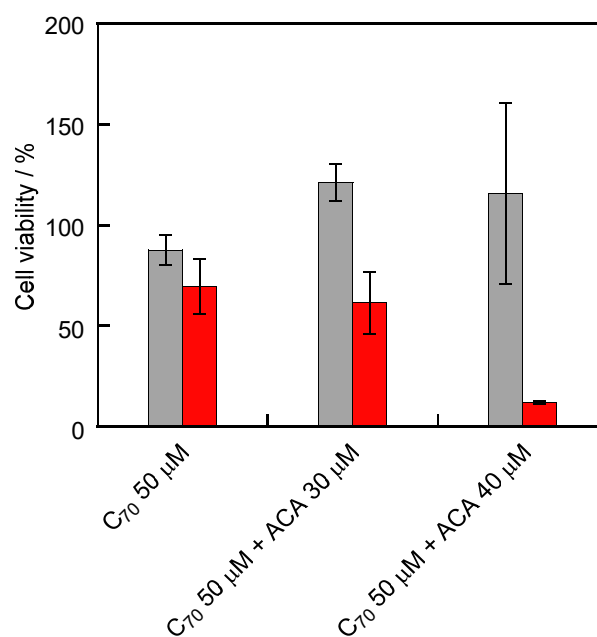
**Fig. 3** Concentration dependency of the cytotoxicities of  $\beta$ -1,3-glucan-complexed  $C_{70}$  in the absence (black line) and the presence of light irradiation (red line) (400–700 nm, 30 min) toward RAW264.7 cells. Each value represents the mean  $\pm$  standard deviation (SD) of three experiments.



**Fig. 4** Time-dependent bleaching of ABDA caused by singlet oxygen generated from  $\beta$ -1,3-glucan-complexed  $C_{70}$  (red line) and  $\gamma$ -cyclodextrin-complexed  $C_{70}$  (black line). Changes in the ABDA absorption at 400 nm upon photoirradiation ( $> 390 \text{ nm}$ ,  $15 \text{ mW cm}^{-2}$ ) were monitored as a function of time ( $\text{Abs}_0$ : initial absorbance).  $[C_{70}] = 15 \mu\text{M}$ ,  $[\text{ABDA}] = 25 \mu\text{M}$ ; under an oxygen atmosphere at  $25^\circ\text{C}$ .

### Drug interaction of $\beta$ -1,3-glucan-complexed $C_{70}$ with ACA

It is well-known that ACA can inhibit the activation of NF- $\kappa$ B, which can be used to regulate the genes that encode proinflammatory cytokines, chemokines, and growth factors.<sup>12</sup> Furthermore, we have confirmed the effect of not only an anti-inflammatory response<sup>13</sup> but also apoptogenic activity<sup>27</sup> of  $\beta$ -1,3-glucan-complexed ACA *in vitro* and *in vivo*. Therefore, it was expected that the photodynamic activity of  $\beta$ -1,3-glucan-complexed  $C_{70}$  can be improved by the addition of  $\beta$ -1,3-glucan-complexed ACA. To investigate the synergistic effect, RAW264.7 cells were co-treated with  $\beta$ -1,3-glucan-complexed ACA and  $50 \mu\text{M}$  of  $\beta$ -1,3-glucan-complexed  $C_{70}$  corresponding to the  $\text{IC}_{50}$  value (Fig. 5).  $\beta$ -1,3-glucan-complexed ACA has no toxicity under dark conditions. In contrast, the photodynamic activity of glucan-complexed  $C_{70}$  was improved in the presence of  $40 \mu\text{M}$  of  $\beta$ -1,3-glucan-complexed ACA. Under these conditions, the cell viability decreased to 10%. This synergistic effect was not observed if  $30 \mu\text{M}$  of  $\beta$ -1,3-glucan-complexed ACA was used. The result indicated that the photodynamic activity of  $\beta$ -1,3-glucan-complexed  $C_{70}$  increases in combination with  $\beta$ -1,3-glucan-complexed ACA because the mechanisms of the toxicity differs between  $\beta$ -1,3-glucan-complexed  $C_{70}$  by a  $^1\text{O}_2$  generation<sup>17</sup> and  $\beta$ -1,3-glucan-complexed ACA by an inhibition of the activation of NF- $\kappa$ B.<sup>13</sup>

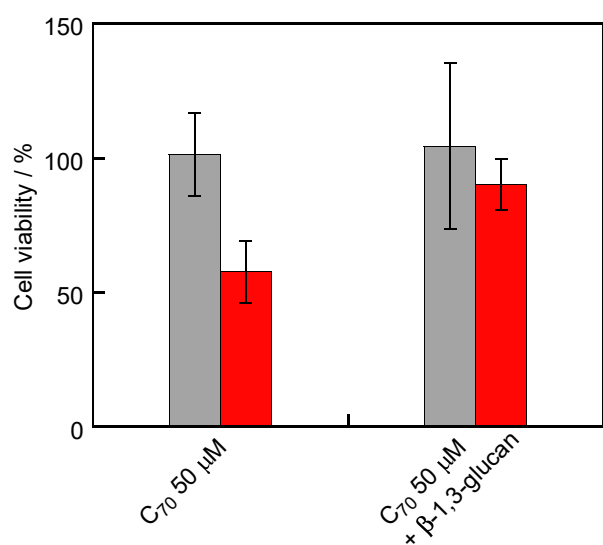


**Fig. 5** Concentration dependence of the cytotoxicities of  $\beta$ -1,3-glucan-complexed  $C_{70}$  and  $\beta$ -1,3-glucan-complexed ACA in the absence (grey bars) and the presence of light irradiation (red bars) (400–700 nm, 30 min) toward RAW264.7 cells. Each value represents the mean  $\pm$  standard deviation (SD) of three experiments.

### Effect of dectin-1 receptor for intracellular uptake of $\beta$ -1,3-glucan-complexed $C_{70}$ by macrophage

First, we investigated the inhibitory effect of  $\beta$ -1,3-glucan for the photodynamic activity of  $\beta$ -1,3-glucan-complexed  $C_{70}$  (Fig. 6). As shown in Fig. 6, the photodynamic activity of  $\beta$ -1,3-glucan-complexed  $C_{70}$  toward RAW264.7 cells decreased after

the addition of  $\beta$ -1,3-glucan. The inhibitory effect of  $\beta$ -1,3-glucan showed that the interaction between  $\beta$ -1,3-glucan and the cell surfaces is of importance for intracellular uptake of  $\beta$ -1,3-glucan-complexed  $C_{70}$  by RAW264.7 cells.

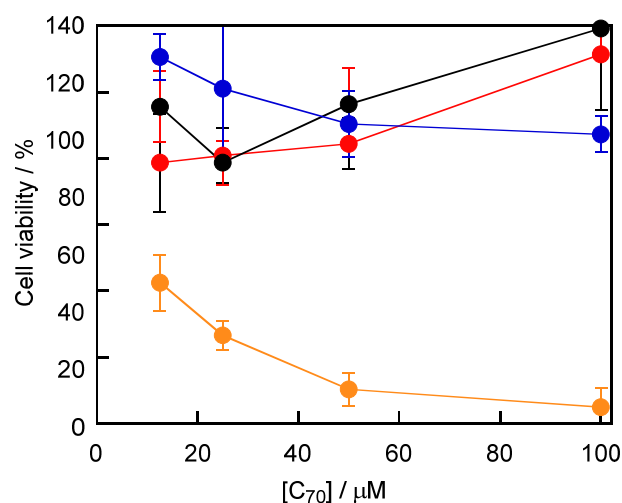


**Fig. 6** Inhibitory effect of  $\beta$ -1,3-glucan (500  $\mu$ g mL<sup>-1</sup>) for the cytotoxicities of  $\beta$ -1,3-glucan-complexed  $C_{70}$  in the absence (grey bars) and the presence of light irradiation (red bars) (400–700 nm, 30 min) toward RAW264.7 cells. Each value represents the mean  $\pm$  standard deviation (SD) of three experiments.

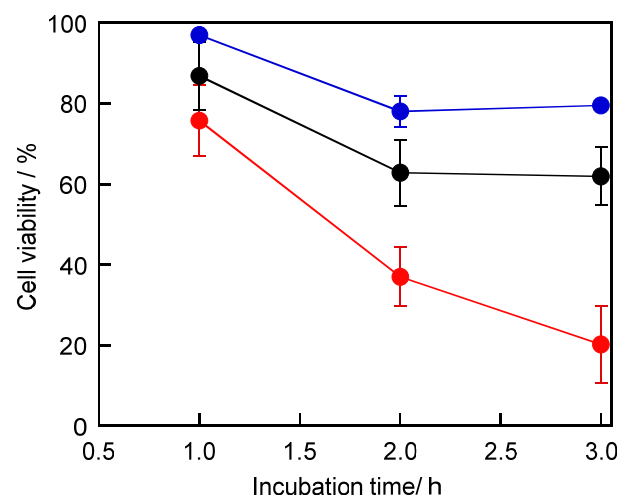
It is well known that dectin-1 acts as a major receptor of  $\beta$ -1,3-glucan on leukocytes. To define the role of dectin-1 in the intracellular uptake and photodynamic activity of  $\beta$ -1,3-glucan-complexed  $C_{70}$ , primary mouse peritoneal macrophages expressing high levels of dectin-1 (hd-M $\Phi$ s) and low levels of dectin-1 (ld-M $\Phi$ s) were prepared. Photocytotoxicity was hardly observed when  $\beta$ -1,3-glucan-complexed  $C_{70}$  was added to ld-M $\Phi$ s. Furthermore, the cell viability did not decrease with  $\beta$ -1,3-glucan-complexed  $C_{70}$  toward hd-M $\Phi$ s under dark conditions. In contrast, the photodynamic activity of  $\beta$ -1,3-glucan-complexed  $C_{70}$  toward hd-M $\Phi$ s was drug dose-dependent and the IC<sub>50</sub> value was approximately 20  $\mu$ M of  $C_{70}$  (Fig. 7). These results indicated that  $\beta$ -1,3-glucan-complexed  $C_{70}$  was internalised by macrophages through the dectin-1 receptor. As shown in Fig. 7, the photodynamic activity of  $\beta$ -1,3-glucan-complexed  $C_{70}$  toward hd-M $\Phi$ s was higher than that toward RAW264.7 cells because of the high expression of dectin-1 on the cell surfaces of hd-M $\Phi$ s. Furthermore, the photodynamic activity toward L929 cells, which hardly express dectin-1 receptor on the cell surfaces, was lower than those toward RAW264.7 cells and hd-M $\Phi$ s (Fig. 8).

To directly evaluate the intracellular uptake of  $\beta$ -1,3-glucan by hd-M $\Phi$ s, fluorochrome-labelled  $\beta$ -1,3-glucan was prepared using  $\beta$ -1,3-glucan-complexed FITC without  $C_{70}$  because  $C_{70}$  acts as a quencher of FITC. The hd-M $\Phi$ s were incubated with a  $\beta$ -1,3-glucan-complexed FITC for 4 h and compared with a dextran-complexed FITC, which is not recognised by dectin-1. As shown in Fig. 9,  $\beta$ -1,3-glucan-complexed FITC accumulated in hd-M $\Phi$ s. In contrast, the intracellular uptake of dextran-complexed FITC was scarcely observed. These results support

the conclusion that hd-M $\Phi$ s interact with  $\beta$ -1,3-glucan-complexed FITC through the dectin-1 receptor.



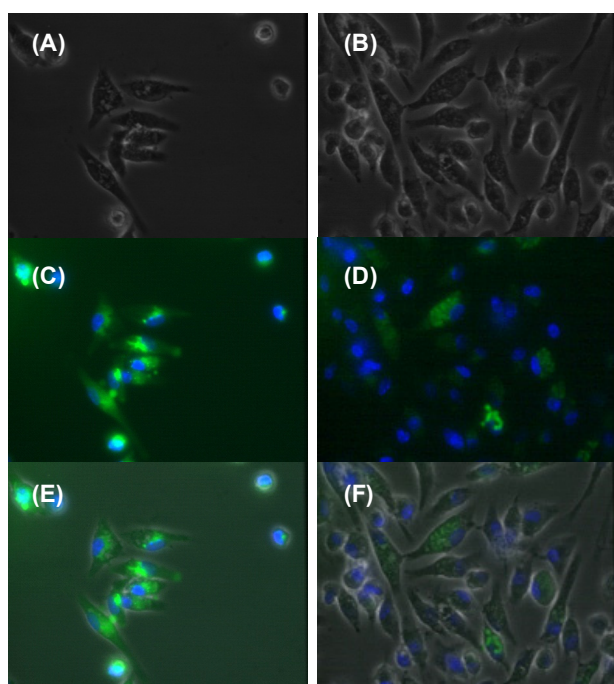
**Fig. 7** Concentration dependency of the cytotoxicities of  $\beta$ -1,3-glucan-complexed  $C_{70}$  in the absence (black and blue lines) and the presence of light irradiation (red and orange lines) (400–700 nm, 30 min) toward ld-M $\Phi$ s (black and red lines) and hd-M $\Phi$ s (blue and orange lines). Each value represents the mean  $\pm$  standard deviation (SD) of three experiments.



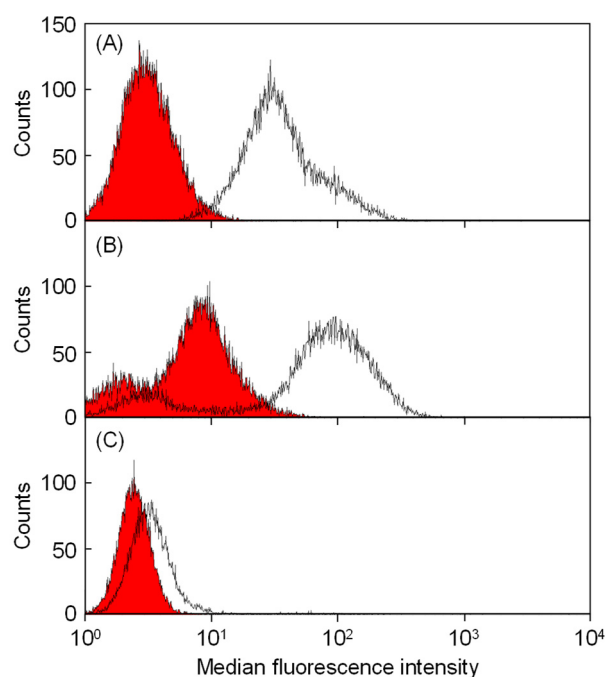
**Fig. 8** Incubation time-dependence of the cytotoxicities of  $\beta$ -1,3-glucan-complexed  $C_{70}$  in the presence of light irradiation (400–700 nm, 30 min) toward RAW264.7 (black line), hd-M $\Phi$ s (red line) and L929 (blue line) cells. Each value represents the mean  $\pm$  standard deviation (SD) of three experiments.

Furthermore, the intracellular uptake of  $\beta$ -1,3-glucan-complexed FITC was quantified by flow cytometric analysis (FACS). The histograms of the fluorescence distribution were plotted as the number of cells versus fluorescence intensity on an algorithmic scale (Fig. 10). As shown in Fig. 10, the intracellular uptake of  $\beta$ -1,3-glucan-complexed FITC by hd-M $\Phi$ s was greater than that by RAW264.7 and L929 cells. The efficiencies of the intracellular uptake were in the following order: hd-M $\Phi$ s > RAW264.7 > L929. This order coincided with the expression levels of dectin-1 on the cells. These results also indicated that  $\beta$ -1,3-glucan incorporated M $\Phi$ s through dectin-1-receptor-mediated endocytic processes.





**Fig. 9** Confocal microscopic images (A and B) and fluorescence microscopic images (C and D) for the hd-MΦs after  $\beta$ -1,3-glucan-complexed FITC (green) (A, C and E) or the dextran-complexed FITC (green) (B, D and F) was applied for 4 h and the cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (blue). Panels E and F are overlaps of A and C and B and D, respectively.



**Fig. 10** Flow cytometry analysis for the detection of  $\beta$ -1,3-glucan-complexed FITC in (A) RAW264.7 cells, (B) hd-MΦs and (C) L929 cells (unfilled histogram). Control indicates autofluorescence of untreated cells (red filled histogram).

## Conclusions

$\beta$ -1,3-Glucan-complexed  $C_{70}$  was prepared using a HSVM apparatus. The intracellular uptakes and photodynamic activities of  $\beta$ -1,3-glucan-complexed  $C_{70}$  decreased in the order of hd-MΦs>RAW264.7>>L929 cells, which reflected the decreasing order of the expression of the dectin-1 receptor on the cell surfaces. These results indicate that the dectin-1 receptor is very important for the photodynamic activity of  $\beta$ -1,3-glucan-complexed  $C_{70}$ . Furthermore, the photodynamic activity was improved by the combination of the  $C_{70}$  complex with  $\beta$ -1,3-glucan-complexed ACA, which has an apoptogenic effect. These findings imply that  $\beta$ -1,3-glucan-complexed  $C_{70}$  shows significant promise for the therapy of macrophage-associated diseases.

## Experimental

### Materials

$\beta$ -(1,3-1,6)-D-Glucan was supplied by Daiso Co. Ltd., (Hyogo, Japan) and was purified.  $C_{70}$  (> 99%) and FITC were purchased from Materials and Electrochemical Research Co. (Tucson, AZ, USA) and Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). The racemic ACA and  $\beta$ -1,3-glucan-complexed ACA were prepared according to our previously reported method.<sup>13</sup>

### Preparation of $\beta$ -1,3-glucan-complexed $C_{70}$

$C_{70}$  (1.40 mg, 1.67  $\mu$ mol), and  $\beta$ -1,3-glucan (10.7 mg) were placed in an agate capsule with two agate-mixing balls. The mixture was mixed vigorously at 25 Hz for 25 min using a high-speed vibration mill (MM 200; Retsch Co., Ltd., Haan, Germany). The solid mixture was suspended in water (4.0 mL) to give a brown emulsion. The mixtures were dissolved in water and the brown emulsion was shaken on a vortex mixer for 1 h. After centrifugation (4500  $\times$  g, 25  $^{\circ}$ C, 20 min), the nondispersed  $C_{70}$  was removed from the solution. The concentration of  $C_{70}$  in the complex with  $\beta$ -1,3-glucan was determined by the molar absorption coefficient for lipid-membrane-incorporated  $C_{70}$  ( $\epsilon_{381} = 4.1 \times 10^4 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$ )<sup>22</sup> instead of that for  $\beta$ -1,3-glucan-complexed  $C_{70}$  (0.27 mM).

### Preparation of $\beta$ -1,3-glucan-complexed FITC

FITC (0.38 mg, 0.98  $\mu$ mol) and  $\beta$ -1,3-glucan (10.1 mg) were placed in an agate capsule with two agate-mixing balls. The mixture was mixed vigorously at 25 Hz for 25 min using a high-speed vibration mill (MM 200; Retsch). The solid mixture was suspended in water (10.0 mL) to give an emulsion. The emulsion was shaken on a vortex mixer for 3 h. After centrifugation (4500  $\times$  g, 25  $^{\circ}$ C, 20 min) and ultrafiltration (6500  $\times$  g, 25  $^{\circ}$ C, 20 min, two times, Microcon 3000), the uncomplexed FITC was removed from the solution.

### Transmission electron microscopy (TEM)

$\beta$ -1,3-Glucan and  $\beta$ -1,3-glucan-complexed  $C_{70}$  were assessed by transmission electron microscopy using a 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 drying in vacuo for 1 h. TEM observations were carried out by using a JEM-2200FS field emission electron microscope (JEOL Ltd., Tokyo, Japan) with an acceleration voltage of 200 kV.

#### Dynamic light scattering (DLS) analysis

The hydrodynamic diameter of  $\beta$ -1,3-glucan-complexed  $C_{70}$  was measured using a Zetasizer (Nano ZS, Malvern Instruments Ltd., Malvern, UK). The instrument consisted of a He/Ne laser operating at 633 nm and 10 mW. DTS Nano version 5.00 (Malvern Instruments Ltd., Malvern, UK) software was used.

#### Cell culture

RAW264.7 and L929 cells were maintained in a  $CO_2$ -independent medium (Gibco BRL, Eggenstein, Germany) supplemented with 10% fetal calf serum at 37 °C in 5%  $CO_2$ . For the experiments conducted to determine the photodynamic activities of  $\beta$ -1,3-glucan-complexed  $C_{70}$ , the cells were seeded in 96-well culture plates at a density of  $5.0 \times 10^4$  cells per well. After growing overnight, the cells were used for the experiments.

Hd-M $\Phi$ s and Id-M $\Phi$ s were prepared as follows. All animal experiments were performed according to the guidelines of Osaka City University. Male C57 BL/6 mice (7 weeks of age) were purchased from Kyudou Co. Ltd. (Kumamoto, Japan). The mice (7–11 weeks of age) received an intraperitoneal (i.p.) injection with 3 mL of thioglycollate medium (3%, Nissui Pharmaceutical, Tokyo, Japan), according to the standard procedure.<sup>28</sup> After 3 days, the elicited peritoneal macrophages were harvested by peritoneal lavage using 6 mL of RPMI-1640 medium (containing 10% FBS and 1 wt % penicillin and streptomycin mixture). The lavage fluids were pooled and centrifuged at 1000 rpm for 5 min. The cell pellet was washed three times with 10 mL of the medium and the cells were seeded into plates and allowed to adhere for 5 h. The cells were washed to remove nonadherent cells and then incubated for 3h and 24 h to prepare Id-M $\Phi$ s and hd-M $\Phi$ s, respectively.

#### Photodynamic activity experiments

The cells were incubated with  $\beta$ -1,3-glucan-complexed  $C_{70}$  for 24 h in the absence of light. The cells were washed with phosphate-buffered saline (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., Osaka, Japan) equipped with a VIS mirror module (300–700 nm) and a long-pass filter with a cut-off at 400 nm. The power of the light at the cellular level was  $9.1 \text{ mW cm}^{-2}$  (400–700 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 a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan), according to the manufacturer's instructions.

#### Generation ability of singlet oxygen

The detection of  $^1O_2$  generation using ABDA (Sigma-Aldrich, Milwaukee WI, USA) was conducted in accordance with methods previously described in the literature.<sup>21,22</sup> The concentrations of  $C_{70}$  in the complexes and ABDA in the mixed solutions were  $[C_{70}] = 15 \text{ }\mu\text{M}$  and  $[ABDA] = 25 \text{ }\mu\text{M}$ . Oxygen was bubbled through all the samples for 30 min prior to their photoirradiation to generate the necessary 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 390 nm. The light was cooled by passing it through a water filter. The power of the light was  $15 \text{ mW cm}^{-2}$  (at 390 nm) at the sample level.

#### Fluorescence microscopy of hd-M $\Phi$ s

The hd-M $\Phi$ s were pre-incubated at 37 °C without  $CO_2$  for 30 min before being treated with  $\beta$ -1,3-glucan- or dextran-complexed FITC. Following a 30-min period of incubation with  $\beta$ -1,3-glucan- or dextran-complexed FITC at 37 °C, the cells were monitored by phase contrast microscopy. The cells were observed using an Olympus IX71 epifluorescence microscope attached to a confocal scan unit CSU-10 (Yokogawa, Tokyo) equipped with a 200 $\times$  objective lens. Phase contrast images and Fluorescence images were recorded with an ORCA-ER CCD Camera (Hamamatsu Photonics, Shizuoka). The following sets of filters were used: DAPI (BP360–370, DM400, and BA420) and FITC (BP460–490, DM500, and BA520).

#### Combination experiments of $C_{70}$ and ACA

RAW264.7 cells were seeded in 96-well culture plates at a density of  $5.0 \times 10^4$  cells per well. After growing overnight, the cells were incubated with  $\beta$ -1,3-glucan-complexed  $C_{70}$  for 24 h in the absence of light. The cells were washed with PBS and then incubated with  $\beta$ -1,3-glucan-complexed ACA for 3 h in the absence of light. After washing with PBS, the cells were exposed to light for 30 min at 25 °C. Light irradiation and the measurement of the viability of cells were performed under the same conditions as mentioned above.

#### Flow cytometry analysis

RAW264.7 cells, hd-M $\Phi$ s and L929 cells were incubated with  $\beta$ -1,3-glucan-complexed FITC for 24 h at 37 °C in 5%  $CO_2$ . After incubation, the cells were washed with PBS, detached with 0.05% trypsin/0.02% EDTA-PBS (EDTA=ethylenediamine-tetraacetic acid), and then suspended in PBS. The suspended cells were added directly to a FACS Calibur flow cytometer. The analysis was gated to include single cells on the basis of forward and side light scattering and was based on the acquisition of data from 10000 cells. The log of the fluorescence was determined and displayed as single-parameter histograms. The geometric mean fluorescence intensity was calculated by using the CellQuest 3.0 program (Becton–Dickinson).

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