because the biodegradable needle-like nanomaterials are considered not to harm the organisms [3, 5]. Hence, the biodegradation properties of C_{60}NWs are required for the biological assessment.

Macrophages are one of the immune system cells and defend the host against the foreign substances in a nonspecific manner during the early phase of infection. THP-1 is a human acute monocytic leukemia cell line and it is well known that the THP-1 cells are induced to differentiate into macrophage-like cells by treatment with PMA [6]. In our previous pilot study, we observed the macrophage-like cells exposed to 0.1, 1 and 10 μg/mL of the C_{60}NWs with the average length of 6.0 μm and the average diameter of 660 nm by an inverted optical phase-contrast microscope for 48 h [7]. The macrophage-like cells were observed to internalize the C_{60}NWs gradually, but the exposed C_{60}NWs didn't affect the cellular morphology. The C_{60}NWs may not exert the affect which is similar to the needle-like structure if macrophages decompose them.

In this study, we estimated the uptake rate of C_{60}NWs by macrophage-like cells in detail and assessed the biodegradability of C_{60}NWs by the cells as one of the biodegradation assessments of the C_{60}NWs in organisms.

2 Materials and Methods

2.1 Materials

2.1.1 C_{60}NWs

C_{60}NWs were synthesized by the liquid-liquid interfacial precipitation method using a C_{60}-saturated toluene solution and isopropyl alcohol [1, 7]. The length of C_{60}NWs ranged from 1 to 17 μm with an average of 6.0 μm and their diameter ranged from 300 to 1340 nm with an average of 660 nm.

2.1.2 Macrophage-like cells

THP-1 cells were purchased from American Type Culture Collection (ATCC, VA, USA). The THP-1 cells were cultured in a RPMI1640 medium (Invitrogen, CA, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS, JRH Biosciences, KS, USA), 100 units/mL penicillin and 100 μg/mL streptomycin (Nacalai Tesque, Japan) (culture solution) at 37°C in an atmosphere of 5% CO_2 and saturated humidity. The THP-1 cells were subcultured every three or four days, where the number of cells in culture was maintained by centrifugation (at 1000 rpm for 3 min) and subsequent resuspension at 2 x 10^5 viable cells/mL. The THP-1 cells were induced to differentiate into macrophage-like cells by treatment with 10 nM of PMA (Wako Pure Chemicals, Japan) for 24 h at 37°C in an atmosphere of 5% CO_2 and saturated humidity [7].

2.2 Methods

2.2.1 Exposure to C_{60}NWs

C_{60}NWs were dispersed in the culture solution with a concentration of 1 mg/mL [7]. The macrophage-like cells were exposed to the C_{60}NWs' suspension with the final concentration of 10 μg/mL C_{60}NWs that was adjusted by ultrasonic agitation.

2.2.2 Phagocytosis assay of C_{60}NWs

2 x 10^5 THP-1 cells were induced to differentiate into macrophage-like cells by PMA on a cover glass (12-545-85, Thermo Fisher Scientific, MA, USA) in 2 mL of culture solution inside a 35 mm polystyrene culture dish (Greiner Bio-One, Germany). The macrophage-like cells were exposed to 20 μL of the C_{60}NWs' suspension. After 1, 3, 6, 12, 24 and 48 h of the exposure, the macrophage-like cells were fixed by 4% paraformaldehyde (Muto Pure Chemicals, Japan) and stained with rhodamine-phalloidin (Sigma-Aldrich, MO, USA) and Hoechst 33342 (Wako Pure Chemicals, Japan). The macrophage-like cells were observed with a differential interference contrast and confocal laser scanning microscope (TCS SP5, Leica Microsystems, Germany) to locate three-dimentionally the position of C_{60}NWs.

2.2.3 Observation of C_{60}NWs in Macrophage-like cells

2 x 10^5 THP-1 cells were induced to differentiate into macrophage-like cells by PMA in 2 mL of culture solution in the 35 mm polystyrene culture dish. The macrophage-like cells were exposed to 20 μL of the C_{60}NWs suspension. Half of the medium was replaced by a new medium (10 nM PMA, 100 μg/mL penicillin, 100 units/mL streptomycin and 10% heat inactivated FBS in RPMI1640) every day for 28 days after the exposure for one day. The macrophage-like cells and C_{60}NWs were observed by an inverted optical phase-contrast microscope (DMIL-HC, Leica Microsystems, Germany) every day before the medium replacement. As a control experiment, the macrophage-like cells that were not exposed to C_{60}NWs and the C_{60}NWs in the PMA-containing medium were observed by the inverted optical phase-contrast microscope every day before the medium replacement.

2.2.4 Observation of the C_{60}NWs after exposure

1 x 10^5 THP-1 cells were induced to differentiate into macrophage-like cells in 1 mL of culture solution

using a cell culture insert (0.4 μm of pore size, Millipore, MA, USA) hanged from the top edge of a 6-well plate (Greiner bio-one, Germany) (Fig. 1). 4 mL medium was used (1 mL in the cell culture insert and the other 3 mL in the 6-well dish). The macrophage-like cells were exposed to 10 μL of the C_{60}NWs suspension. 0.5 mL of PMA-containing medium was poured into the cell culture insert after removing 0.5 mL of old medium from the 6-well plate every day for 28 days. Immediately and 28 days after the exposure, the macrophage-like cells were decomposed by 4 mL of proteinase K (Wako Pure Chemicals, Japan) with a concentration of 200 μg/mL at 50°C for 3 h after washing the cells twice with 4 mL of PBS buffer. The C_{60}NWs were washed with 4 mL of ultrapure water twice on the membrane of cell culture insert. The change of C_{60}NWs was observed with an optical microscope (ECLIPSE ME 600, Nikon, Japan) to measure the length. The morphological change of C_{60}NWs was observed with a scanning electron microscope (SEM, JSM-6700, JEOL, Japan) after coating the membrane of cell culture insert with Pt for 1 min by a deposition apparatus (ESC-101, ELIONIX, Japan). C_{60}NWs were dispersed in a PMA-containing medium as a control experiment. The change of C_{60}NWs was similarly observed as above.

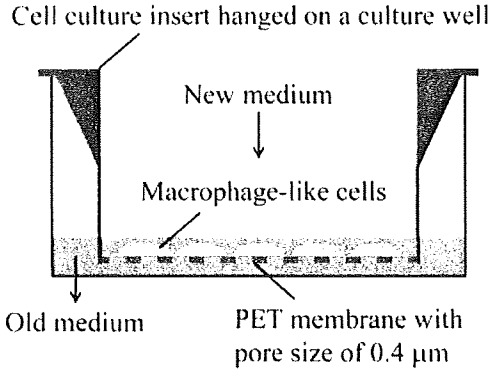

Fig.1. Macrophage-like cells were cultivated on a PET membrane with C_{60}NWs.

3 Results

3.1 Phagocytosis assay of C_{60}NWs

As shown in Fig. 2, the C_{60}NWs were phagocytized by the macrophage-like cells. The macrophage-like cells internalized the C_{60}NWs with time and more than 70% of the cells internalized them after 48 h exposure to 10 μg/mL of C_{60}NWs (Fig. 3).

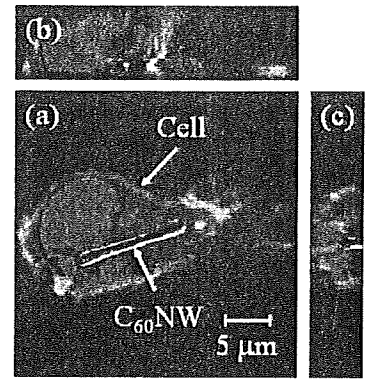

Fig.2. Confocal laser microscopy images with differential interference contrast of the macrophage-like cells exposed to the C_{60}NWs for 24 h. (a) Horizontal cross section, (b) and (c) vertical cross sections. The nucleus and F-actin are shown in blue (Hoechst 33342) and in red (rhodamine-phalloidin), respectively. The Hoechst 33342 was excited with light of 405 nm wavelength and the emission was monitored at 420-520 nm. The rhodamine-phalloidin was excited at 543 nm and the emission was monitored at 560-700 nm.

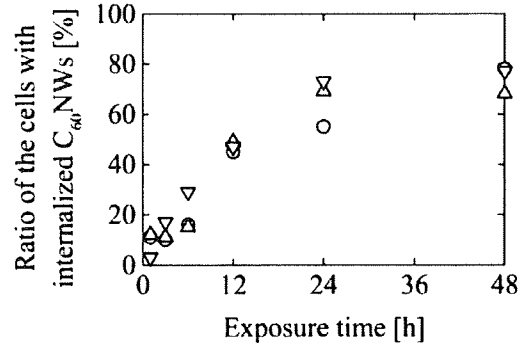

Fig.3. Ratio of the macrophage-like cells with internalized C_{60}NWs. 100 macrophage-like cells were observed for each point.

3.2 Biodegradation assessment of C_{60}NWs

After the long-term co-culture of macrophage-like cells and C_{60}NWs, decomposed C_{60}NWs were observed in the cells (Fig. 4).

A change of length distribution of C_{60}NWs was estimated (Fig. 5). The number of short (less than 3.0 μm in length) C_{60}NWs increased after the co-culture with the macrophage-like cells for 28 days (Fig. 6). In contrast, at the control experiment, an increase of the

number of short C_{60}NWs was not observed.

The change of C_{60}NWs' morphology was not observed in the medium for 28 days (Fig. 7). On the other hand, granular crystals were observed on the membrane after the co-culture of macrophage-like cells and C_{60}NWs for 28 days.

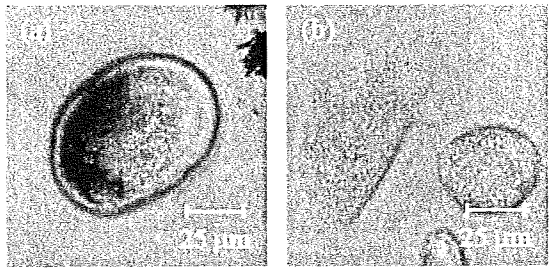

Fig.4. (a) Macrophage-like cells cultivated (a) with and (b) without C_{60}NWs for 21 days after the exposure to C_{60}NWs.

4 Discussion

4.1 Uptake of C_{60}NWs

Macrophages have a role to recognize, internalize and digest foreign materials. The uptake of foreign materials depends on their size and surface properties [8]. C_{60} is phagocytized by macrophages [9] and the uptake rate of C_{60} is lower than that of graphite particles [10].

The C_{60}NWs were also phagocytized by macrophage-like cells and the macrophage-like cells internalized the C_{60}NWs with time and more than 70% of the cells internalized them after 48 h of exposure to 10 μg/mL of C_{60}NWs. However, in our previous study, no alteration of cellular morphology was observed in the macrophage-like cells exposed to C_{60}NWs [7]. The macrophage-like cells were able to internalize the C_{60}NWs without their alteration of cellular morphology.

4.2 Biodegradation of C_{60}NWs

After the long-term co-culture of macrophage-like cells and C_{60}NWs, decomposed C_{60}NWs were observed in the cells and the number of short (less than 3.0 μm in length) C_{60}NWs increased. In addition, the change of C_{60}NWs' morphology was observed after the co-culture with the macrophage-like cells. It is unlikely that these observed substances were composed of the materials derived from the culture medium and washing buffer, because a sufficient amount of water was used for the final wash of C_{60}NWs after the treatment with the enzyme in order to decompose the macrophage-like cells and these

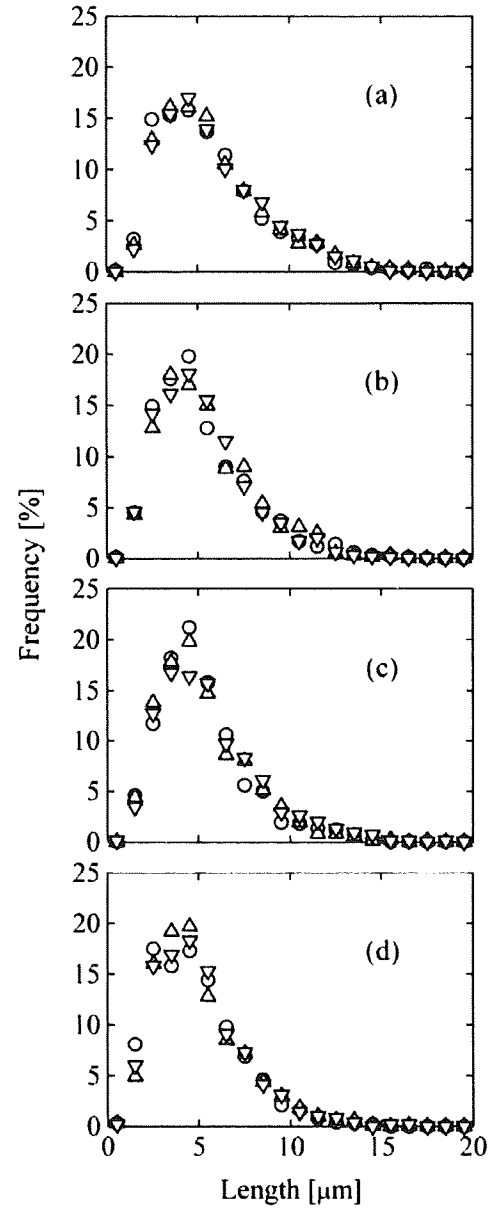

Fig.5. Length distribution of C_{60}NWs. (a) immediately after the exposure of culture medium to C_{60}NWs, (b) immediately after the exposure of macrophage-like cells to C_{60}NWs, (c) 28 days after the exposure of culture medium to C_{60}NWs and (d) 28 days after the exposure of macrophage-like cells to C_{60}NWs. The length was measured by an optical microscope after the enzymatic treatment and washing on the cell culture insert. Each symbols were expressed by measuring the length of about 1000 C_{60}NWs.

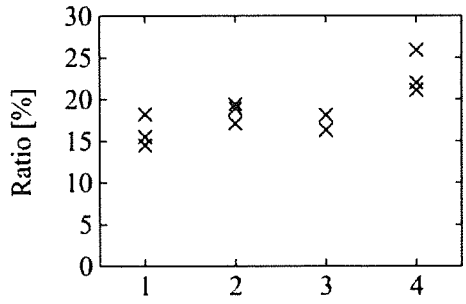

Fig.6. The ratio of short (less than 3.0 μm in length) C_{60}NWs. 1: Immediately after the exposure of culture medium to C_{60}NWs (Fig. 5 (a)). 2: Immediately after the exposure of macrophage-like cells to C_{60}NWs (Fig. 5 (b)). 3: 28 days after the exposure of culture medium to C_{60}NWs (Fig. 5 (c)). 4: 28 days after the exposure of macrophage-like cells to C_{60}NWs (Fig.5 (d)). Each point was expressed by measuring the length of about 1000 C_{60}NWs.

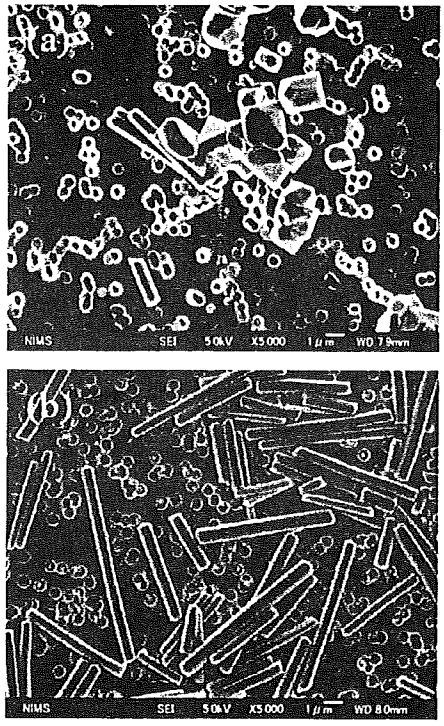

Fig.7. SEM images of the substances on the cell culture insert after the 28 days' exposure of (a) the macrophage-like cells and (b) the culture medium to C_{60}NWs.

substances were not observed at the control experiment. Hence, it is suggested that these substances are composed of fullerene molecules derived from the C_{60}NWs. It is considered that the macrophage-like cells decompose C_{60}NWs into individual C_{60} molecules and that those observed granular substances must have recrystallized from these C_{60} molecules via a dissolution-recrystallization process during the long-term co-culture or upon the enzymatic treatment.

These results suggest that the C_{60}NWs may decompose into individual C_{60} molecules by macrophages owing to the weak van der Waals bonding forces acting between the C_{60} molecules of C_{60}NWs. On the basis of this assumption, the C_{60}NWs may exert the effect which is not similar to that of the needle-like structure but is similar to that of fullerene molecules on organisms. Previous studies have reported that C_{60} (the aggregate size was not described or larger than 1 μm) were nontoxic against mammalian cells [10, 11, 12]. The C_{60}NWs may also be nontoxic against organisms. Hence, the C_{60}NWs are expected for various applications not only in the engineering fields but also in the biological field such as drug delivery systems and tissue engineering.

In this study, we demonstrated that the macrophage-like cells decompose C_{60}NWs. However, the mechanism is not clear. Recent studies show human neutrophils generate not only reactive oxygen species but also ozone in bacterial killing and inflammation [13, 14]. Additionally, there has been considerable research on the THP-1 [15]. We are going to carry out further research on the biodegradation mechanism of C_{60}NWs by the macrophage-like cells and on the biological impact (cell viability, LDH, cytokines, active oxygen and ozone generation, and so on) of C_{60}NWs using short and long C_{60}NWs.

5 Conclusion

The interaction between macrophage-like cells and C_{60}NWs was investigated in this study. Macrophage-like cells were exposed to 10 μg/mL of C_{60}NWs with an average length of about 6.0 μm and an average diameter of 660 nm. The macrophage-like cells internalized the C_{60}NWs with time and more than 70% of the cells internalized the C_{60}NWs after 48-h exposure. After the long-term co-culture, decomposed C_{60}NWs were observed in the macrophage-like cells and the number of short (less than 3.0 μm in length)

C_{60}NWs increased after the exposure. These results suggest that macrophages can decompose C_{60}NWs into individual C_{60} molecules as the primary immune response.

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