Biodegradation of C₆₀ Fullerene Nanowhiskers by Macrophage-like Cells

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Abstract: To evaluate the biological impact of C₆₀ fullerene nanowhiskers (C₆₀NWs), an interaction between phorbol 12-myristate 13-acetate (PMA)-treated THP-1 cells (macrophage-like cells) and the C₆₀NWs was investigated in this study. The macrophage-like cells were exposed to 10 μg/mL of C₆₀NWs with an average length of about 6.0 μm and an average diameter of 660 nm. After 1, 3, 6, 12, 24 and 48 h of the exposure, the cells were fixed, stained with Hoechst 33342 and rhodamine-phalloidin and were observed by a differential interference contrast and confocal laser scanning microscope to estimate an uptake rate of C₆₀NWs into cells. To assess the biodegradability of C₆₀NWs by the macrophage-like cells, the cells and the exposed C₆₀NWs were observed by an inverted optical phase-contrast microscope for 28 days after the exposure. After the long-term co-culture of cells and C₆₀NWs, the cells were decomposed by proteinase K and the exposed C₆₀NWs were observed with an optical microscope and a scanning electron microscope to examine the change of C₆₀NWs by the cells. The macrophage-like cells internalized the C₆₀NWs with time and more than 70% of the cells internalized the C₆₀NWs after 48-h exposure. After the long-term co-culture, decomposed C₆₀NWs were observed in the cells and the number of short (less than 3.0 μm in length) C₆₀NWs increased after the exposure. These results suggest that macrophages may be able to decompose C₆₀NWs into C₆₀ molecules as the primary immune response.

Key-Words: Fullerene nanowhisker, Needle-like crystal, Biodegradation, Macrophage, Biological assessment, In vitro

1 Introduction
Nanomaterials possess enormous potential for wide application in various fields owing to their unique properties and some of them have already been used in daily life. Fullerene nanowhiskers (FNWs), one of the most promising nanomaterials, have needle-like structures, and are composed of the fullerene molecules that are usually bonded via van der Waals forces and are synthesized by the liquid-liquid interfacial precipitation method [1]. The FNWs are expected for various applications such as low-dimensional semiconductors, field emission tips, nanoprobes for microdevices, fiber-reinforced nanocomposites, composite elements for lubrication, and so on. But the biological impact of FNWs is not clear and should be studied before their practical use.
Carbon nanotubes (CNTs), one of the most promising nanomaterials, have also the needle-like structure like FNWs. Long CNTs may be hazardous to health and environment owing to their needle-like morphology and biopersistence like asbestos [2, 3]. The nanosized needle-like structure resembling asbestos has been suspected to induce the asbestososis via inhalation. Recent studies demonstrated that multiwalled carbon nanotubes (MWCNTs) reached the subpleura in mice after the inhalation administration of MWCNTs [4]. By the exposure of mesothelioma lining of the body cavity of mice to MWCNTs, an asbestos-like pathogenic behavior associated with CNTs was observed, indicating a structure-activity relationship based on the length, to which asbestos and other pathogenic fibers show [2].
It is important to know whether the needle-like nanomaterials are decomposed in organisms or not,
because the biodegradable needle-like nanomaterials are considered not to harm the organisms [3, 5]. Hence, the biodegradation properties of C₆₀NWs are required for the biological assessment.

Macrophages are one of the immune system cells and defend the host against the foreign substances in a non-specific 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 C₆₀NWs 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 C₆₀NWs gradually, but the exposed C₆₀NWs didn’t affect the cellular morphology. The C₆₀NWs 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 C₆₀NWs by macrophage-like cells in detail and assessed the biodegradability of C₆₀NWs by the cells as one of the biodegradation assessments of the C₆₀NWs in organisms.

2 Materials and Methods
2.1 Materials
2.1.1 C₆₀NWs
C₆₀NWs were synthesized by the liquid-liquid interfacial precipitation method using a C₆₀-saturated toluene solution and isopropyl alcohol [1, 7]. The length of C₆₀NWs 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% CO₂ 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 10⁵ 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% CO₂ and saturated humidity [7].

2.2 Methods
2.2.1 Exposure to C₆₀NWs
C₆₀NWs were dispersed in the culture solution with a concentration of 1 mg/mL [7]. The macrophage-like cells were exposed to the C₆₀NWs’ suspension with the final concentration of 10 μg/mL C₆₀NWs that was adjusted by ultrasonic agitation.

2.2.2 Phagocytosis assay of C₆₀NWs
2 x 10⁵ THP-1 cells were induced to differentiate into macrophage-like cells by PMA in 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 C₆₀NWs’ 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-dimensionally the position of C₆₀NWs.

2.2.3 Observation of C₆₀NWs in Macrophage-like cells
2 x 10⁵ 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 C₆₀NWs 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 C₆₀NWs 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 C₆₀NWs and the C₆₀NWs 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 C₆₀NWs after exposure
1 x 10⁵ 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 C₆₀NWs 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 C₆₀NWs were washed with 4 mL of ultrapure water twice on the membrane of cell culture insert. The change of C₆₀NWs was observed with an optical microscope (ECLIPSE ME 600, Nikon, Japan) to measure the length. The morphological change of C₆₀NWs 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). C₆₀NWs were dispersed in a PMA-containing medium as a control experiment. The change of C₆₀NWs was similarly observed as above.

Fig. 1. Macrophage-like cells were cultivated on a PET membrane with C₆₀NWs.

3 Results
3.1 Phagocytosis assay of C₆₀NWs
As shown in Fig. 2, the C₆₀NWs were phagocytized by the macrophage-like cells. The macrophage-like cells internalized the C₆₀NWs with time and more than 70% of the cells internalized them after 48 h exposure to 10 μg/mL of C₆₀NWs (Fig. 3).

Fig. 2. Confocal laser microscopy images with differential interference contrast of the macrophage-like cells exposed to the C₆₀NWs 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.

3.2 Biodegradation assessment of C₆₀NWs
After the long-term co-culture of macrophage-like cells and C₆₀NWs, decomposed C₆₀NWs were observed in the cells (Fig. 4). A change of length distribution of C₆₀NWs was estimated (Fig. 5). The number of short (less than 3.0 μm in length) C₆₀NWs 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.

![Figure 4](image)

**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 $\mu$g/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 $\mu$m 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

![Figure 5](image)

**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)
C₆₀NWs increased after the exposure. These results suggest that macrophages can decompose C₆₀NWs into individual C₆₀ molecules as the primary immune response.

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