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In situ observation of $C_{60}(C(COOH)_2)_2$ interacting with living cells using fluorescence microscopy

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Abstract The interactions between nanoparticles and living cells were investigated by an imaging technique of fluorescence microscopy. For this purpose, the C_{60} derivative $C_{60}(C(COOH)_2)_2$, a therapeutic agent for degeneration diseases of central nervous system, was synthesized, purified and characterized. Its interaction with the living cell and penetration of the cellular membrane were *in situ* studied using the real time imaging technique, and its potential cytotoxicity was also examined by flow cytometry. The results indicate that $C_{60}(C(COOH)_2)_2$ can easily enter cells, and is mainly located in cytoplasm by fluorescein labeling. Furthermore, $C_{60}(C(COOH)_2)_2$ can carry the molecule that cannot cross cellular membranes into cells, because fluorescein compound itself cannot enter the cell or adhere to membrane. At concentrations ranging from 1×10^{-2} to 1×10^{2} mg/L, $C_{60}(C(COOH)_2)_2$ does not show any detectable cytotoxicity.

Keywords: fluorescence microscopy, real time imaging, fullerene derivative $C_{60}(C(COOH)_2)_2$, cross cellular membrane, fluorescence labeling.

1 Introduction

In recent years, studies on biological effects of the nanoscale materials have become the cornerstone of rapidly developed nanomedical and nanobiological technologies. Moreover, studies on the bio-effects when the different kinds of nanoscale materials enter human body have attracted a lot of interest of scientists and formed a very important field of the frontiers of sciences[1−8]. The mechanism of nanoparticles interacting with cells is one of the most important issues which need to be studied. However, to explore the process of cells taking up nanoparticles is restricted by the current experimental technologies. Fullerene C_{60}, due to its unique structure, has attracted considerable attention in biology applications[9−11] since its discovery. The applications of a number of water-soluble C_{60} derivatives have been reported so far[12−15]. More and more chemists, biologists and pharmacologists are devoted to this field due to the exciting medical activities of water-soluble C_{60} derivatives. Because of their favorable biological and cellular activities, C_{60} carboxyl derivatives are among the most widely studied C_{60} derivatives. As a neuroprotective agent, tris C_{60} malonic acid derivative is used to inhibit the degeneration of dopaminergic neurons in Parkinson's disease[16]. The experiment showed that after treatment with C_{60} malonic acid derivative, mice suffering from the Lou Gehrig's disease lived about 10 d longer than those had not been treated with[17].

Could C_{60} derivatives enter cells? If yes, what is the mechanism? What are their intracellular receptors? The small sizes of C_{60} derivatives make it possible that they can penetrate the biomembrane and enter cells, or even organelles, such as mitochondrion, endoplasmic reticulum, golgis apparatus, and nucleus. In the very beginning, Scrivens *et al.*[18] found that C_{60} became cell-associated rapidly. They proposed that the C_{60} particles may be associated with the cell surface followed by diffusion of molecular C_{60} or its aggregated particles. By the means of indirect immunofluorescence, Sarah *et al.*[19] demonstrated that water-soluble C_{60} mono-malonic acid derivative could cross the lipid bilayer and help other molecules enter cells. In this paper, the C_{60} derivative $C_{60}(C(COOH)_2)_2$ is synthesized, purified and characterized. Its interaction with the living cell and penetration of the cellular membrane are studied *in situ* using the real time imaging fluorescence technique, and its potential cytotoxicity is also examined by flow cytometry.

2 Experiment

2.1 Synthesis of $C_{60}(C(COOH)_2)_2$

Bis-adducts of C_{60} water soluble di-malonic acid derivative $C_{60}(C(COOH)_2)_2$ were synthesized as described by Lamparth and Hirch[20] combining with the separating and purifying performance[21,22]. The synthesis method was basically according to the classic Bingle-Hirsch reaction[23,24], a reaction between C_{60} and diethyl bromomalonate under the basical circumstance, which was commonly used to produce malonyl adducts of fullerenes. Briefly, NaH was added to the solution of C_{60} in toluene, and the solution became dark red from purple at the beginning. And then diethyl bromomalonate was added. After stirring under argon protection and vacuum for 10 h, the solution was filtered to remove the precipitation, and the solvent was removed in vacuum. The residue was separated by chromatography on silica gel (400 mesh) using different mobile phases, in order of toluene, toluene-hexane (1∶1), and hexane. There were six fractions obtained, corresponding to the different C_{60} malonic ester derivatives, respectively. The fraction VI, eluted by toluene was dissolved in dried toluene, and then 20 folder excessive NaH was added. The solution was stirred under argon at 80℃ for 10 h. After the heating source was removed, CH_3OH was added to stop the reaction, and then HCl was added. The solution was cooled in air. Precipitate was collected by centrifugation, and was washed with CH_3OH, HCl, and H_2O twice, respectively. The resulting precipitate was dissolved in the water and filtrated with membrane filter with a pore size of 0.22 μm. Finally, the resulting filtrate was dried in vacuum. The brown dried powder was the expected compound 1, $C_{60}(C(COOH)_2)_2$.

2.2 Synthesis of fluorescently labeled $C_{60}(C(COOH)_2)_2$

Two hundred μL 1-ethy-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 2.5 mg 5-(5-aminopentyl) thioureidyl) fluorescein dihydrobromide salt (5-FITC cardaverine) were added into the 200 μL water solution of compound 1, of which 4 mg $C_{60}(C(COOH)_2)_2$ was already dissolved. After resulting in dark at the room temperature for 4 h, the final green-yellow solution was applied on a Sephadex G-15 column using highly purified distilled water as eluent. Fluorescently labeled $C_{60}(C(COOH)_2)_2$ was obtained as compound 2.

2.3 Characterization

The mass spectrum of $C_{60}(C(COOEt)_2)_2$ was analyzed by matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS, Autoflex, Bruker Co, Germany). The radical characterization of $C_{60}(C(COOH)_2)_2$ was obtained on infra-red spectrometer (AVATAR360, Nicolet, USA).

2.4 Cell culture

HeLa and Rh35 cells were grown in a Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% foetal calf serum (FCS), penicillin (100 μg/mL), and streptomycin (100 μg/mL) in a 37℃, 5% CO_2 atmosphere humidified incubator.

2.5 Fluorescence imaging of cells incubated in $C_{60}(C(COOH)_2)_2$

Exponentially growing HeLa cells were transferred into three Petri dishes, respectively, and incubated in dark at 37℃, 5% CO_2 for 48 h, which made the cells adhere to the bottom of dishes. Before incubation, the culture medium was removed, and 1 mL DMEM without serum was dropped into the Petri dishes. And then, 5-FITC cardaverine was added to one of them, and as control, group 0, fluorescence labeled $C_{60}(C(COOH)_2)_2$ was added to the other two, as groups 1 and 2. Groups 0 and 1 were incubated at 37℃, 5% CO_2 for 2 h, group 2 for 30 min in the same condition. After that, cells were washed with PBS buffer three times, for removing the fluorescence and fullerene molecules, which did not enter the membrane adhibiting the cell surface. Fluorescence images were taken immediately after the incubation and washing steps. The excitation wavelength of the argon ion laser and the magnification of the fluorescence microscope were set to 488 nm and 60×, respectively.

2.6 Flow cytometry and cytoxicity assay

5 mL 10^5/mL of cells was incubated in a culture flask，and 24 h later, the original culture medium was replaced by 5 mL culture medium without serum. Different concentrations of $C_{60}(C(COOH)_2)_2$ were added to cell medium at 1×10^{-2} mg/L, 1×10^{-1} mg/L, 1 mg/L, 1×10^{1} mg/L, 1×10^{2} mg/L, respectively, without $C_{60}(C(COOH)_2)_2$ as control. After 24 h incubation, cells were harvested, washed with physiological saline solution twice, and fixed with 70% ethanol at 4℃. The cell

suspension was supplemented with 25 mg/L RNase A and 50 mg/L propidium iodide (PI) prior to the measurement, after washing with physiological saline solution twice and suspending. PI would not stain live cells. It would enter dead or late apoptotic cells and incorporate into DNA, thereby selectively staining the dead and late apoptotic cells into red color. The amount of apoptosis cells would be measured by the hypo-diploid peak which appeared beside the G1 peak.

3 Results and discussion

The MALDI-TOF mass spectral analyses of $C_{60}(C(COOEt)_2)_2$ sample are shown in Fig. 1 with peaks at m/z = 720 and 1036. According to the molecular weight, it could be concluded that C_{60} di-malonic ester derivative $C_{60}(C(COOEt)_2)_2$ (m/z = 1036) can dissociate into pristine C_{60} (m/z = 720) and bis-adduct under the laser excitation processes. The hydrolysate of $C_{60}(C(COOH)_2)_2$ was characterized by infrared spectroscopy and is shown in Fig. 2, which conformed with the early method[20]. Fig. 2 provides some structural information. First, there is a very strong peak of –OH absorption at 3422.91 cm^{-1}, with COO-absorption of 1715.92 cm^{-1} and a very broad peak in the region. Another strong peak at 1403.27 cm^{-1} is O-H vibration frequency, also with 1095.12 cm^{-1} of C-O stretching absorptions. Signals which could be seen clearly in this area indicate the presence of carboxyl acid group in $C_{60}(C(COOH)_2)_2$.

Recently, observation of interaction of fullerenes

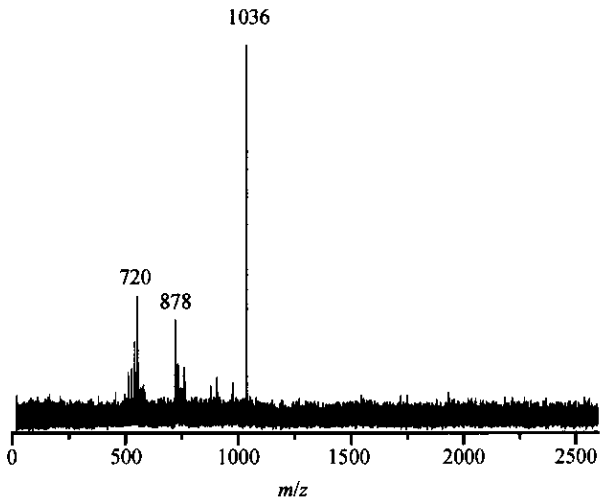

Fig. 1. MALDI-TOF mass spectra of $C_{60}(C(COOEt)_2)_2$, negative ion and reflection modes.

with cells by laser confocal microscopy[25] was reported. However, it was demonstrated that cell fixation, even under mild conditions, might lead to the artifactual redistribution of $C_{60}(C(COOH)_2)_2$ mainly into other organelle [26,27], which brought the infidelity of result. As the fact that there was no obvious evidence that $C_{60}(C(COOH)_2)_2$ translocated across the cell membrane after 30 min incubation, as shown in experiment group 2, it was concluded that the event of $C_{60}(C(COOH)_2)_2$ entering cells was a time and energy consumed procedure. Fig. 3(a) shows the image of fluorescence labeled $C_{60}(C(COOH)_2)_2$ (green fluorescence) in cells after being incubated for 2 h. Because C_{60} molecules were apt

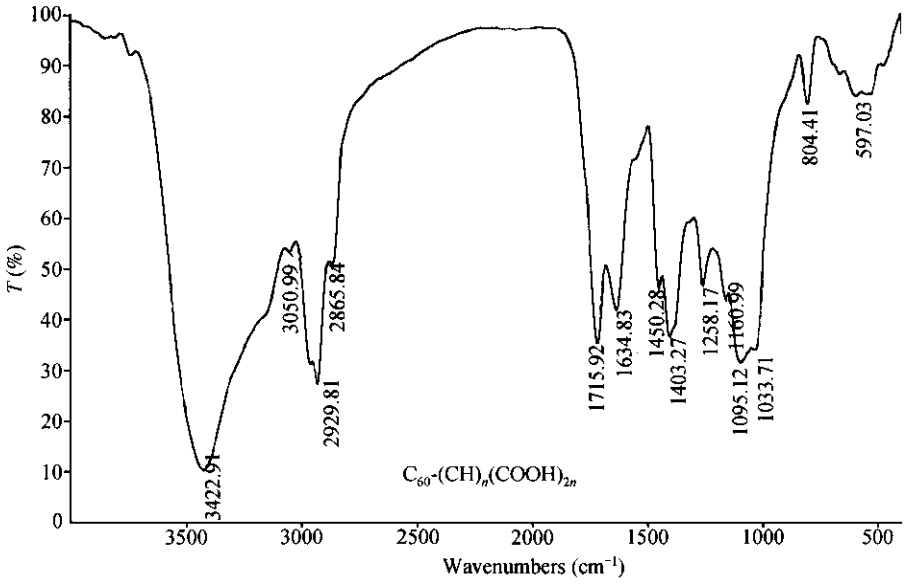

Fig. 2. IR spectrum of $C_{60}(C(COOH)_2)_2$.

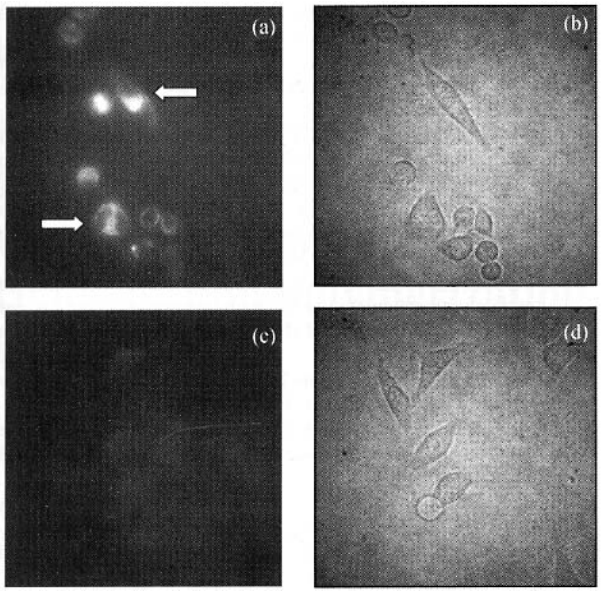

Fig. 3. (a) Experiment group, image of fluorescence labeled $C_{60}(C(COOH)_2)_2$ in cells; (b) bright field image of (a); (c) control group, image of cells which identified 5-FITC cardaverine would not enter cells alone; (d) bright field image of (c).

to adhere onto the cell membrane surface, it was generally hard to identify the exact localization of them. The fluorescence appearing inside the cells on division stage (indicated by white arrows in Fig. 3(a) was the best proof that $C_{60}(C(COOH)_2)_2$ could enter cells. It is worthy to note that due to the sensitivity of fluorescence microscopic techniques, if there is only single or several fluorescent molecules in any cellular components, the fluorescence signals could be too weak to be observed. These mean that quite an amount of nanoparticles have entered the living cells. Moreover, the result cannot exclude the possibility of $C_{60}(C(COOH)_2)_2$ localizing in other organelles besides cytoplasm. Fig. 3(c) shows the result of control group cells incubated with 5-FITC cardavarine for 2 h, and no fluorescence was observed. Thus, $C_{60}(C(COOH)_2)_2$ molecules are apt to enter cells, and mainly localize in the cytoplasm. They can deliver the fluorescein that cannot enter cells alone to cross cell membranes. This implies that it should be a promising carrier system for drug delivery and targeting therapy. According to the experiment results and the discussion about the mimic structures between the fullerene cage and that of the clathrin by Sarah *et al*.[19], a conclusion obtained was different from that of Scrivens *et al*.[18]. Based on the properties of size, structure, and lipotropy, $C_{60}(C(COOH)_2)_2$ molecules will be easily attracted by the negative charge cell membrane surfaces with boundary clathrins, and be able to cross cell membranes in the endocytosis pathway by clathrin-coated vesicles. Besides endocytosis, other biological pathway could be possible, including some ion channels, except human ether-a-go-go-related gene (HERG) K^+ channel[28]. The mechanism of cells taking up C_{60} derivatives may be different according to the variety of modification groups, which needs not only further researches but also some development for effective methodology.

To identify the potential cytotoxicity of $C_{60}(C(COOH)_2)_2$, flow cytometry and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) toxicity test were used. By cytometry, PI staining method was used to identify dead Rh35 cells. The concentrations of $C_{60}(C(COOH)_2)_2$ delivered was 1×10^{-2}—1×10^{2} mg/L, no appreciable cell death was observed and was similar to the control group as shown in Fig. 4(a). The proportions of apoptosis Rh35 cells are about 2.0%—3.5%, and there is no significant difference observed in histogram. Cycle phase analysis showed that incubation with different concentrations of $C_{60}(C(COOH)_2)_2$ did not affect cell cycle distribution, as S% is 25%—31%, G2/G1 is 1.89—1.94 in Fig. 4. $C_{60}(C(COOH)_2)_2$ showed that there exists also no toxic effect on Rh35 cells at the concentrations of 1×10^{-2} to 1×10^{2} mg/L, so did human liver carcinoma cells (HepG2). The same results of toxic evaluation were obtained by MTT method.

In conclusion, water-soluble bis-adducts C_{60} malonic acid derivative $C_{60}(C(COOH)_2)_2$ could enter cells, and mainly localize in cytoplasm. $C_{60}(C(COOH)_2)_2$ translocating across the cell membrane after incubation for 30 min indicated that the event of $C_{60}(C(COOH)_2)_2$ entering cells is a time and energy consumed procedure. Although fluorescein molecules cannot enter cells by themselves, they can readily transport across cell membranes and enter cells when conjugating to $C_{60}(C(COOH)_2)_2$ with no appreciable toxicity at the concentrations ranging from 1×10^{-2} to 1×10^{2} mg/L. It may provide new insights for drug delivery and targeting therapy applications. According to these results and studies on mono-adduct C_{60} malonic acid derivative[19], it is concluded that the efficiency of C_{60} delivering system can be enhanced by increasing the number of the modification groups. The present work established a new method for studying how nanomaterials enter cells. The results suggest that the fluorescence microscopy imaging technique is a practical means to directly observe the processes of nanomaterials interacting with cells in the biological environment.

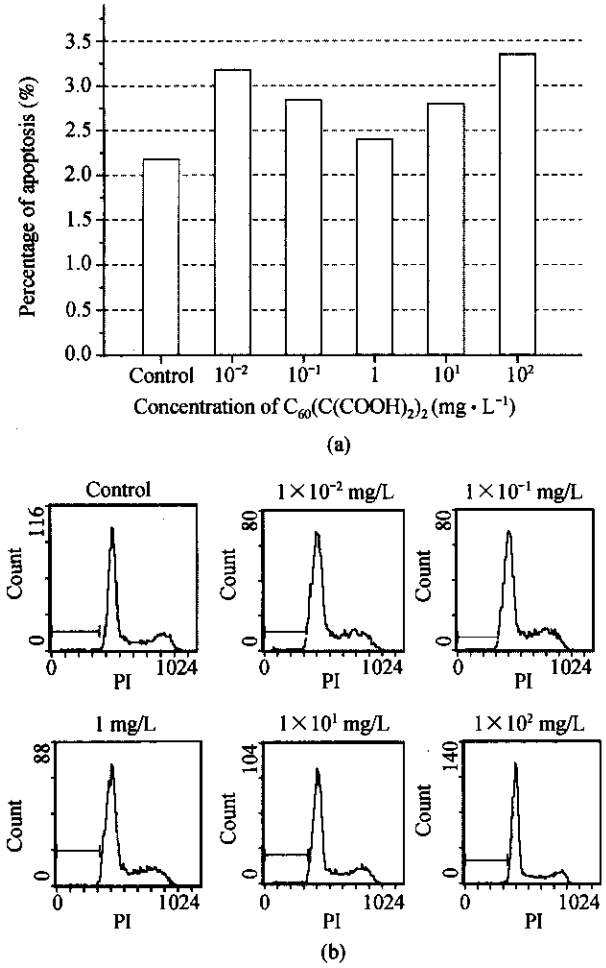

Fig. 4. Evaluation of the potential cytotoxicity of $C_{60}(C(COOH)_2)_2$ by flow cytometry (a) bar graph of percentage of Rh35 cell apoptosis at the concentration of 1×10^{-2} mg/L, 1×10^{-1} mg/L, 1 mg/L, 1×10^{1} mg/L, 1×10^{2} mg/L, and untreated group with $C_{60}(C(COOH)_2)_2$ as control; (b) cell apoptosis histogram.

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