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Preparation and Cellular Uptake of pH-Dependent Fluorescent Single-Wall Carbon Nanotubes

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Abstract: Fluorescent single-wall carbon nanotubes (SWCNTs) were prepared by mixing cut SWCNTs with acridine orange (AO). The optical absorbance and fluorescence characteristics of AO–SWCNT conjugates display interesting pH-dependent properties. Fluorescence microscopy in combination with transmission electron microscopy proves that AO–SWCNTs can enter HeLa cells and are located inside lysosomes. The endocytosis-inhibiting tests show that the clathrin-mediated endocytosis is a key step in the internalization process. The internalized AO–SWCNTs remain inside lysosomes for more than a week and have little effect on cell proliferation. These findings may be useful in understanding the SWCNT-based intracellular drug delivery mechanism and help to develop new intracellular drug transporters.

Keywords: acridine orange · carbon nanotubes · endocytosis · lysosomes

Introduction

Due to their remarkable and unique physical, chemical, and physiological properties, single-wall carbon nanotubes (SWCNTs) have attracted considerable interest in a variety of areas including biomedical applications.[1,2] It has been shown that SWCNTs can serve as highly efficient delivery vehicles to transport a wide range of molecules such as DNA, proteins, and other drug molecules across membranes into living cells.[3–7] In addition, the intrinsic stability and structural flexibility of SWCNTs may prolong the circulation time as well as the bioavailability of drug molecules conjugated to SWCNTs.[8–11]

Though it has been demonstrated that SWCNTs are capable of entering cellular membranes, the entry mechanism is still under debate. Kam et al.[12] and Jin et al.[13] reported that protein- or single-stranded oligonucleotide-modified SWCNTs could enter cells by means of endocytosis. Kostarelos et al.[7] suggested an energy-independent nonendocytotic mechanism involving insertion and diffusion of SWCNTs through different cellular barriers. In addition, a drug delivery system requires inert auxiliary and body-friendly and biodegradable excipients.[11] However, because SWCNTs are chemically stable and non-biodegradable, they might come in direct contact with cells and tissues after release of transported drug. The long-term effect of SWCNTs after their uptake by cells should be carefully considered before they can be used as biological transporters. Therefore, fluorescent SWCNTs with low cytotoxicity need to be designed and prepared to clarify these fundamental issues.

In this work, acridine orange (AO) was chosen as a marker for SWCNTs. Noncovalently bound AO–SWCNTs conjugates were prepared by mixing AO with cut SWCNTs in an aqueous suspension by means of electrostatic interaction and π–π stacking. Successful formation of AO–SWCNTs was confirmed by high-resolution transmission electron microscopy (HRTEM). The AO–SWCNTs thus obtained show interesting distinct pH-dependent properties. The AO–SWCNTs have almost no fluorescence in a neutral environment but can emit green fluorescence in acidic solutions, and their intensity increases as the pH value decreases. Therefore AO–SWCNTs would be expected to display obvious fluorescence upon entry into acidic organelles such as lysosomes. By use of the lysosomotropic dye LysoTracker Red and TEM, we demonstrated that AO–SWCNTs could enter HeLa cells and are located inside the lysosomes; there they showed green fluorescence. The results of endocytosis-inhibiting experiments showed that entry of AO–SWCNTs into HeLa cells was predominated by clathrin-mediated endocytosis. Long-term tracking of AO–SWCNTs suggested that AO–SWCNTs could remain inside lysosomes for more than a week and had little effect on cell proliferation.

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Results and Discussion

The raw, as-grown SWCNTs tend to cluster into large bundles (see Figure S1a in the Supporting Information) so that they have very limited solubility in common solvents. Moreover, some metal particles are usually included in the bundles of SWCNTs and thus can pose considerable biological toxicity. To eliminate these concerns, the SWCNTs used in this study are cut and purified. The cut and purified SWCNTs are short (usually <500 nm), well separated, and only form very small bundles as characterized by TEM (see Figure S1b in the Supporting Information). During this process, many carboxylic groups are generated on the surface of SWCNTs, and these enhance the dispersion of SWCNTs in aqueous solution.[14] In fact, the cut SWCNTs can be dispersed in aqueous solution, thus forming a stable suspension (Figure S2).

Figure 1a shows the scheme used for preparing the AO–SWCNT conjugates. AO–SWCNT conjugates are also well dispersed in water. (see Figure S2 in the Supporting Information). TEM observations show that the cut SWCNTs are very smooth and do not contain any impurities (Figure 1b), thereby indicating the complete removal of metal particles and amorphous carbon. Adsorption of the AO molecules onto SWCNTs led to roughening of the surfaces of SWCNTs, and additional coating layers appeared (Figure 1c, white arrows for AO). In addition, the zeta potential of SWCNTs changed from -62.6 to -33.4 mV after modification with AO, thus indicating that the positively charged AO has been successfully attached to the negatively charged cut SWCNTs due to electrostatic interactions in combination with π–π stackings.[15]

Once fluorescent AO is attached onto SWCNTs, the interaction of these two components is concerted. In a solution of phosphate-buffered saline (PBS) at pH 7.4, AO has an absorption peak at 492 nm, whereas the AO–SWCNT peak blueshifts to 475 nm and shows notable broadening (Figure 2a). It is also shown that the green fluorescence of AO–SWCNTs is negligible in comparison to that of pure AO excited at 475 nm at pH 7.4 (Figure 2b; and Figure S3 in the Supporting Information). These results suggest that there are strong interactions between AO and SWCNTs. As in the case of other noncovalently bonded preparations of fluorescent SWCNTs,[16,17] the energy and electrons from the excitation of AO might directly flow into

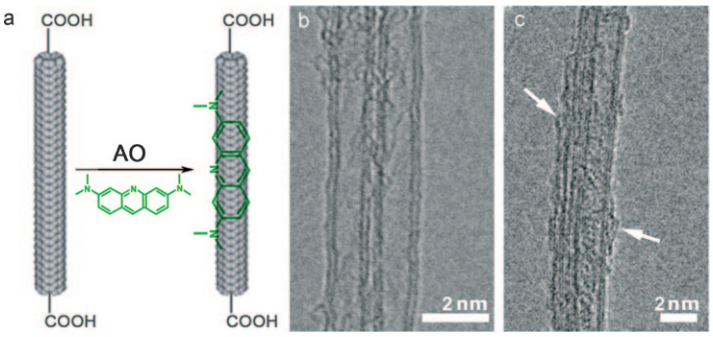

Figure 1. a) Scheme for preparation of AO–SWCNT conjugates. HRTEM images of b) cut SWCNTs and c) AO–SWCNTs.

SWCNTs and thus quench the fluorescence of AO. Interestingly, by lowering the pH values, the UV/Vis absorption of AO–SWCNTs becomes stronger and results in a continuous redshift (Figure 2c); the green fluorescence intensity also increases (Figure 2d and Figure S3 in the Supporting Information). These results indicate that the conjugated material has distinct pH dependence. It appears that the acidic solution weakens the interactions between AO and SWCNTs, thus a few AO molecules can even detach from the side walls of SWCNTs at a very low pH. It can be validated by the presence of free AO molecules in the filtrate after removal of the AO–SWCNTs at pH 2 (see Figure S4 in the Supporting Information). Since the AO is a weak base and SWCNT–COOH is a weak acid, the pH-dependent properties of AO–SWCNTs may result from the acid–base character of AO and SWCNT–COOH.

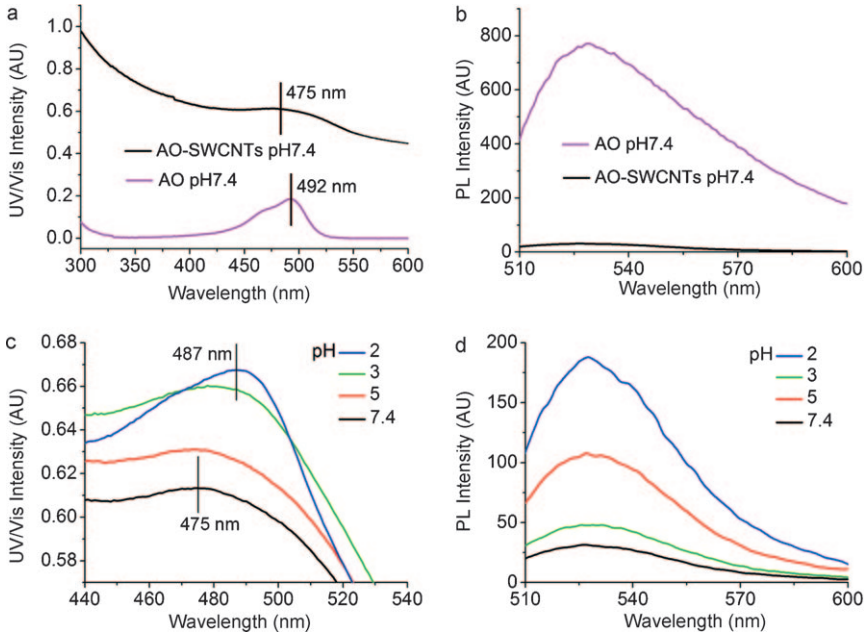

Figure 2. a) UV/Vis and b) photoluminescence (PL) spectra of pure AO ($2\,\mu g\,mL^{-1}$) and AO–SWCNTs ($20\,\mu g\,mL^{-1}$ SWCNT concentration) in PBS at pH 7.4. c) UV/Vis and d) PL spectra of AO–SWCNTs in PBS at different pH values.

TEM and fluorescence microscopy were used to track the distribution of AO–SWCNTs inside the cells. TEM observations show that AO–SWCNTs are internalized by HeLa cells (Figure 3a and b). After incubating HeLa cells with AO–SWCNTs (20 $\mu g\,mL^{-1}$) at 37 °C for 30 min, green fluorescence mainly concentrates in the cytoplasm by using a blue exciting light (Figure 3d), which is well in accordance with the TEM results. If the cells are incubated with only free AO (2 $\mu g\,mL^{-1}$), green fluorescence is observed to be concentrated in the nuclei (see Figure S5 in the Supporting Information). This is probably due to the fact that AO is a cell-permeable nucleic acid selective fluorescent cationic dye, and it can bind to DNA to emit green fluorescence and bind to RNA to then emit red fluorescence. LysoTracker Red is a fluorescent probe for labeling and tracking acidic organelles such as lysosomes in live cells and can emit red fluorescence. When cells stained with LysoTracker Red were incubated with AO–SWCNTs, red fluorescence can be clearly observed (Figure 3e). We also used green exciting light to irradiate the cells containing only AO–SWCNTs. Very little red fluorescence could be observed inside cytoplasm (see Figure S6 in the Supporting Information), which could be due to very little AO detached from SWCNTs and bound to the cytoplasm RNA. Thus, the red fluorescence in Figure 3e is emitted from LysoTracker Red and gives the distribution of lysosomes. The fluorescent regions in Figure 3d are in good agreement with those in Figure 3e, in which a yellow-orange color inside cells can be seen by overlapping them (Figure 3f. This provides direct evidence that the internalized AO–SWCNTs are located inside the acidic lysosomes and can emit green fluorescence as a result of the decrease in pH value.

Endocytosis is known as a general entry mechanism for various extracellular materials and is an energy-dependent process. In contrast, a nonendocytotic mechanism is independent of energy. To investigate the entry mechanism of SWCNTs into cells, we performed several endocytosis-inhibition experiments. It is known that adenosine triphosphate (ATP) production is disrupted when cells are cultured at 4 °C or with NaN_3.[7,12] Our results showed that there was no difference for HeLa cells when cultured with AO at 4 or 37 °C (see Figure S5 in the Supporting Information), and in both cases the green fluorescence is concentrated in the nucleus. However, when HeLa cells were cultured at 4 °C then treated with AO–SWCNTs (Figure 4b), or cultured at 37 °C with NaN_3 (Figure 4c) and then treated with AO–SWCNTs, there was very weak green fluorescence in the cells for both situations. These phenomena were in good agreement with an energy-dependent endocytotic mechanism.

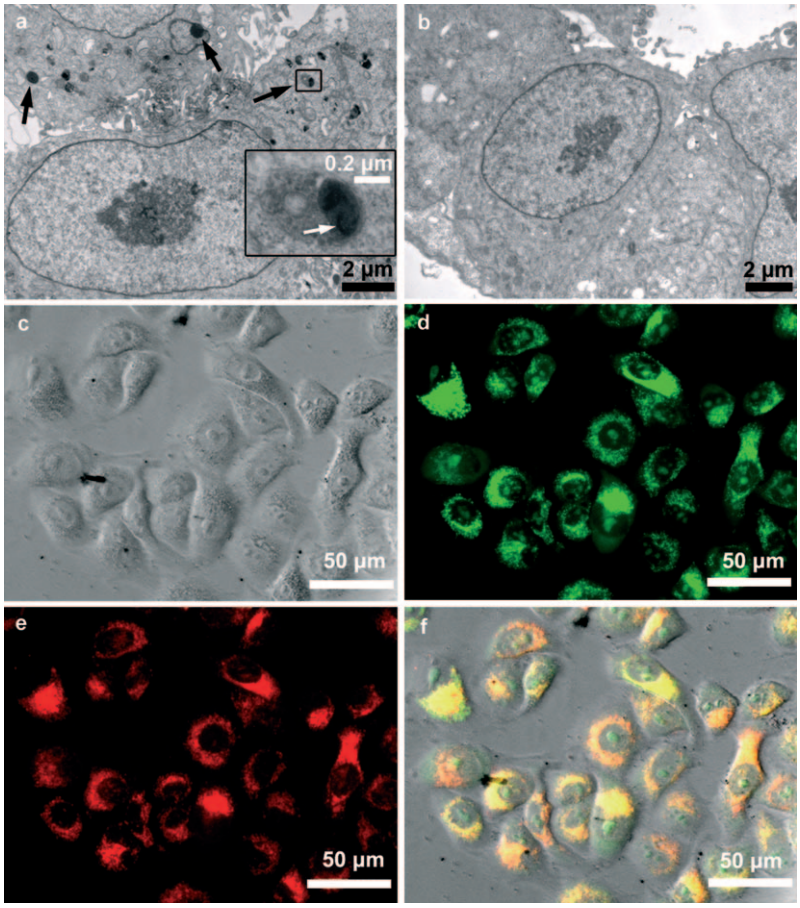

Figure 3. The distribution of AO–SWCNTs inside Hela cells. TEM images of Hela cells a) cultured with AO–SWCNTs (20 $\mu g\,mL^{-1}$ SWCNT concentration) and b) cultured without SWCNTs. Inset: Magnified images of the boxed region in (a). c) Bright field image of HeLa cells incubated with AO–SWCNTs (20 $\mu g\,mL^{-1}$ SWCNT concentration) for 30 min. Corresponding fluorescent images of cells excited by d) blue light, e) green light, and f) overlapping images of (c), (d), and (e). The cells were first stained with a LysoTracker Red marker.

It has been reported that cells can internalize materials by several endocytotic processes such as clathrin-mediated endocytosis, caveolae-mediated endocytosis, macropinocytosis, and so on. Therefore, chlorpromazine (an inhibitor for clathrin-mediated endocytosis), nystatin (an inhibitor for caveolae-mediated endocytosis), and cytochalasin D (an inhibitor of macropinocytosis)[18] were also used. It can be seen in Figure 4d that green fluorescence in the cells is very weak when the cells are pretreated with chlorpromazine. However, the cells pretreated with nystatin (Figure 4e) and cytochalasin D (Figure 4f) remained basically the same compared to the con-

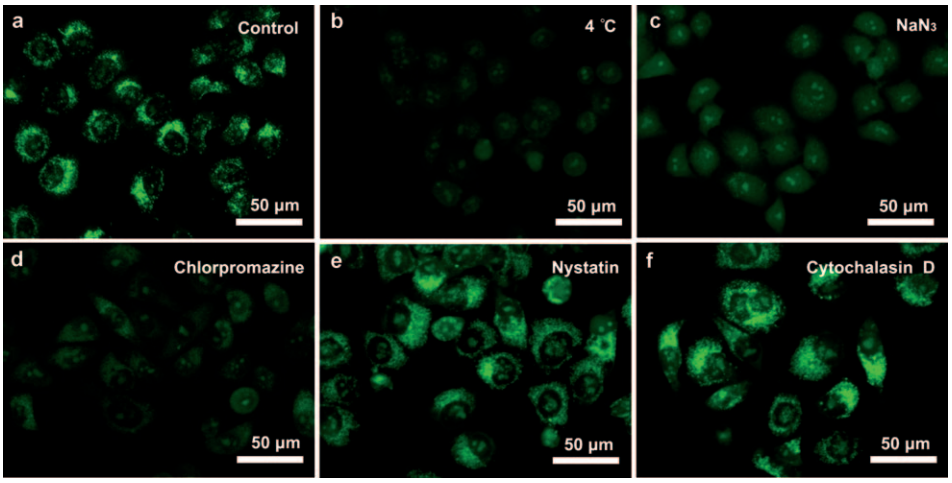

Figure 4. Fluorescence images of HeLa cells incubated with AO–SWCNTs (10 μg mL^{-1} SWCNT concentration) under several endocytosis-inhibiting conditions.

trol sample (Figure 4a). Based on the above experimental results, we propose that the entry of AO–SWCNTs into acidic lysosomes is predominated by clathrin-mediated endocytosis and AO is a reliable maker for tracking this process.

Before long-term monitoring of the distributions of internalized SWCNTs in HeLa cells, the cytotoxicity of cut SWCNTs and their effect on cell proliferation was first examined. The cells were incubated with cut SWCNTs for 1 h at 37 °C, and then washed with fetal bovine serum (FBS)-free Dulbecco's modified eagle medium (DMEM), and cultured with fresh medium containing 5 % FBS for the indicated times. It was shown that the cut SWCNTs can also be internalized by cells (Figure 5a) and their amount was remarkably reduced after 7 days of culturing (Figure 5b). The WST-1 assay was used to assess cell viability after culturing with the cut SWCNTs.[11,19] The results show that there is no apparent cell loss after incubation with the cut SWCNTs, even at the concentration of 100 μg mL^{-1} (Figure 5c), thus suggesting that the internalized SWCNTs have little influence on cellular proliferation. A recent study has reported that SWCNTs could be expelled from cells by means of exocytosis.[13] However, we are not certain whether exocytosis occurs because the increased cell amount could also lead to a decrease in the amount of SWCNTs inside individual cells.

We further used AO–SWCNTs to monitor SWCNTs in living cells after a long period of culturing. HeLa cells were initially cultured with AO–SWCNTs (20 μg mL^{-1} SWCNT concentration) at 37 °C for 1 h, and then cultured in DMEM containing 5 % FBS (Figure 6a). A monolayer of confluent cells was formed after culturing for 7 days, and green fluorescent aggregates could still be observed in the cytoplasm in some cells (Figure 6b). However, after 12 days, most of these disappeared (Figure 6c). TEM images further revealed that the internalized SWCNTs were still present in some cells after culturing for 7 days (Figure 6d), but the amount was remarkably reduced in most cells after culture over a long period of time (Figure 6e). These TEM results are in good agreement with those obtained from the fluorescence studies. It is worth noting that in the case of cells initially cultured with AO,

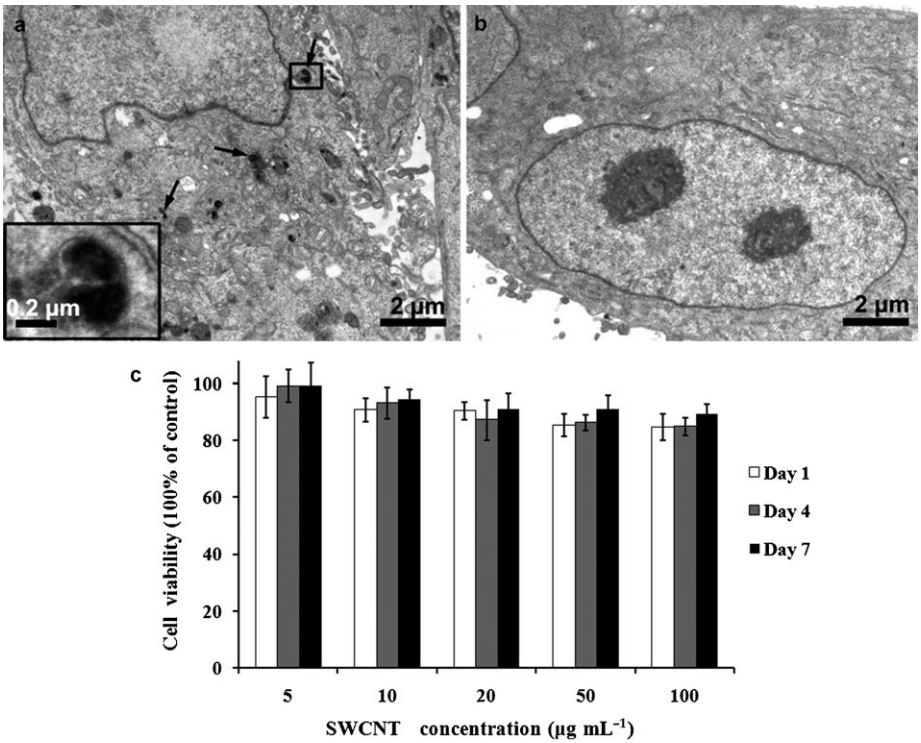

Figure 5. TEM images of a) HeLa cells incubated with cut SWCNTs (20 μg mL^{-1}) for 1 h and b) after continued culturing for 7 days. c) Viability of HeLa cells after incubation with 5–100 μg mL^{-1} cut SWCNTs for 1 h, then continued culturing for 4 and 7 days. Cells cultured without SWCNTs at the same time intervals were used as control (100 % cell viability). Inset: The magnified image of the boxed region in (a).

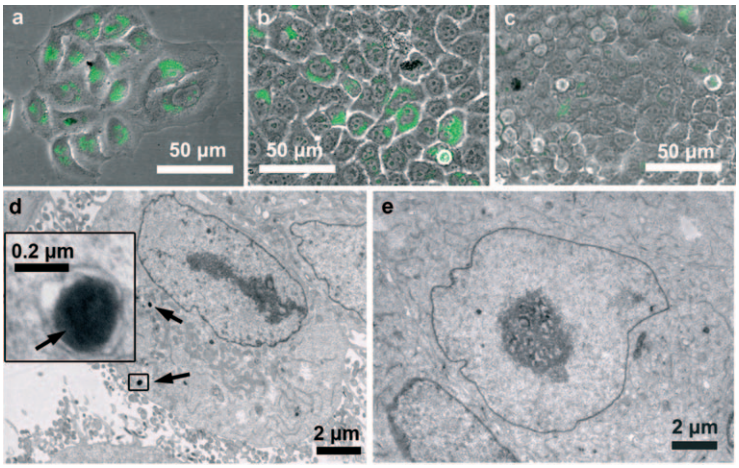

Figure 6. Long-term monitoring of AO–SWCNTs inside cells. a) Merged image of the bright field image and fluorescence image of HeLa cells incubated with AO–SWCNTs (20 μg mL^{-1} SWCNT concentration) for 1 h, and b) the AO–SWCNT-treated cells with continued culturing for 7 days and c) 12 days. TEM images of AO–SWCNT-treated HeLa cells after culturing for d) 7 days and e) 12 days. Inset: Magnified image of the boxed region in (d).

the green fluorescence almost completely disappeared after culturing for 3 days (see Figure S7 in the Supporting Information).

Conclusion

In conclusion, we have demonstrated that noncovalent conjugation of a common dye, AO, to SWCNTs can provide a fluorescent label for monitoring the localization of SWCNTs. AO–SWCNTs display interesting pH-dependent optical properties. They can emit green fluorescence only in an acidic environment, and the fluorescence and absorption intensities increase as the pH value decreases. Based on this phenomenon, AO–SWCNTs have been successfully used to monitor the entry mechanism of the SWCNTs into the HeLa cells. The endocytosis-inhibition results suggest that modified SWCNTs entered acidic lysosomes in HeLa cells by means of a clathrin-mediated endocytosis mechanism. Long-term monitoring of the internalized SWCNTs revealed that the SWCNTs remain in the lysosomes for at least more than one week and have almost no effect on cell proliferation. These findings might be useful in understanding the mechanism of nanomaterial–cell interactions and helpful in developing new SWCNT-based intracellular drug transporters.

Experimental Section

Materials and instruments: SWCNTs were purchased from Chengdu Organic Chemistry Co., Ltd (purity >90%, length >50 μm, diameter 1–2 nm). AO and cytochalasin D were purchased from Sigma. Fetal bovine serum (FBS) and high-glucose Dulbecco's modified eagle medium (DMEM) were obtained from Hyclone. WST-1 reagent, NaN_3, and LysoTracker Red were purchased from Beyondtime Bio-Tech. Chlorpromazine and nystatin were obtained from Dr. Ehrenstorfer GmbH. The porous poly(vinylidene chloride) (PVDC) membrane was obtained from Shanghai ANPEL Instrument Co. Ltd (0.22 μm pore size).

High-resolution transmission electron microscopy (HRTEM) was conducted using a JEOL TEM-2100 at 200 kV. Transmission electron microscopy (TEM) was carried out using a CM120 (Philips, Holland). Zeta potentials were measured using a Zetasizer Nano ZS90 analyzer (Malvern). The UV/Visible (UV/Vis) absorption spectra were recorded using a Lambda 20 spectrometer (Perkin–Elmer). The photoluminescence (PL) spectra were carried out using a luminescence spectrometer (Perkin–Elmer). The fluorescent images were recorded by using an inverted fluorescence microscope (IX 71, Olympus) and a charge-coupled device (CCD, Cascade 650).

Cutting and purification of SWCNTs: The cutting and purification of SWCNTs was essentially carried out using modified literature procedures.[14] Commercial SWCNTs (500 mg) were added into a mixture of H_2SO_4 (98%) and HNO_3 (65%; v/v=3:1, 200 mL) and exposed to sonic irradiation at 0°C for 24 h. The cut SWCNTs were then thoroughly rinsed with ultrapure water (18.2 MΩ) and filtered through a microporous filtration membrane (Φ=0.22 μm). They were redispersed in HNO_3 (2.6 M, 200 mL) and heated at reflux for 24 h, collected by vacuum filtration, and washed several times with ultrapure water to neutrality. The product was then dried under vacuum at 50°C for 24 h for further use.

Modification of SWCNTs with AO: The cut SWCNTs (50 mg) were mixed with AO (5 mg) in phosphate-buffered saline (PBS; 0.01 M, pH 7.4, 50 mL). The mixture was sonicated for 10 min, stirred overnight at room temperature, and dialyzed against ultrapure water in a 12–14 K MWCO membrane at room temperature for one week to remove free AO.

Incubation of living cells with modified SWCNTs: Human cervical carcinoma HeLa cells were cultured in DMEM supplemented with 10% FBS at 37°C in a humidified incubator (MCO-15AC, Sanyo) in which the CO_2 level was kept constant at 5%. Cells and nanotube suspensions were incubated as follows: Cells were cultured overnight to allow cell attachment and then washed with FBS-free DMEM. AO–SWCNT or cut SWCNT suspensions were then added and the resulting mixture was left to incubate at 37°C for 30–60 min. The SWCNT concentration in the incubation culture was typically 10–20 μg mL^{-1}. After incubation, the cells were washed with sterilized PBS several times before further examination under a fluorescence microscope.

Cell staining with AO or LysoTracker Red: For free AO stained cells, the cells were incubated with 2 μg mL^{-1} AO in FBS-free DMEM for 5 min, then washed several times with PBS, and investigated by using a fluorescence microscope. Lysosomotropic dye LysoTracker Red was employed to mark the locations of lysosomes inside cells. The cells were pretreated with the lysotracker in FBS-free DMEM (v/v=1:20000) for 2 h at 37°C, washed several times with PBS to remove the excess dye, then incubated with 20 μg mL^{-1} (SWCNT concentration) AO–SWCNTs for 30 min at 37°C, washed with PBS again, and investigated by fluorescence microscopy.

Endocytosis-inhibition experiments: To investigate the mechanism that determines how AO–SWCNTs enter cells, a series of endocytosis-inhibiting experiments were carried out. HeLa cells were first pretreated with one of the endocytosis inhibitors (0.05% NaN_3 for depletion of adenosine triphosphate; 10 μg mL^{-1} chlorpromazine as inhibitor for clathrin-mediated endocytosis; 15 μg mL^{-1} nystatin as inhibitor for caveolae-mediated endocytosis; and 1 μg mL^{-1} cytochalasin D as inhibitor for macropinocytosis) in FBS-free DMEM for 1 h, and then incubated with AO–SWCNTs for another 30 min. Or the cells were directly incubated with AO–SWCNTs at 4°C for 30 min to deplete adenosine triphosphate. The SWCNT concentration of AO–SWCNTs was 10 μg mL^{-1} in all incubation tests. After incubation, the cells were rinsed three times with 0.01 M PBS prior to examination by fluorescence microscopy.

Tracking cut SWCNTs or AO–SWCNTs in cells by TEM: HeLa cells were seeded in a culture dish with a diameter of 60 mm (Corning). The cells were cultured overnight to allow cell attachment, then incubated with AO–SWCNTs or cut SWCNTs (20 μg mL^{-1} SWCNT concentration)

in FBS-free culture medium for 1 h. The cells were then rinsed with sterilized PBS and cultured in fresh culture medium containing 5% FBS for 7 or 12 days. The culture medium was replaced with fresh medium on alternate days throughout the duration of the experiment. For the TEM analysis, HeLa cells were washed with PBS, and then fixed with 2% glutaraldehyde and 1% osmium tetroxide for 2 h at 4°C. The cells were then dehydrated in a graded ethanol series (30, 50, 70% with 3% uranyl acetate, 80, 95, and 100%) for 10 min at each concentration followed by two changes in 100% propylene oxide. After infiltration and embedding in epoxy resins at 60°C for 48 h, the sections were stained with lead citrate and investigated by TEM.

Cell-viability test: A WST-1 assay was used to measure cell viability. HeLa cells were seeded into a 24-well flat culture plate (Corning). After cell attachment, the cells were incubated with a specific concentration of SWCNTs (5, 10, 20, 50, or 100 $\mu g\,mL^{-1}$) in FBS-free culture medium at 37°C for 1 h. They were then rinsed three times with sterilized PBS and cultured with fresh medium containing 5% FBS for the preset time. Following this, the cells were washed with PBS, and 500 μL PBS was used as a substitute for the culture medium before adding 1:10 (v/v) of the WST-1 reagent. After incubation for another 2 h at 37°C, the absorbance was measured at 450 nm using a microplate reader (Model 680, Bio-Rad). Cells cultured without SWCNTs at the same time intervals were used as the controls.

Acknowledgements

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