Published in final edited form as:

ACS Nano. 2012 December 21; 6(12): 10486–10496. doi:10.1021/nn302457v.

Single-Walled Carbon Nanotubes Alter Cytochrome C Electron Transfer and Modulate Mitochondrial Function

Xiaowei Ma^{†,‡}, Li-Hua Zhang^{†,§,‡}, Li-Rong Wang^{†,‡}, Xue Xue[†], Ji-Hong Sun^{§,*}, Yan Wu[†], Guozhang Zou[†], Xia Wu^{†,§}, Paul C. Wang[#], Wayne G. Wamer[&], Jun-Jie Yin[&], Kaiyuan Zheng[†], and Xing-Jie Liang^{†,*}

[†]Laboratory of Nanomedicine and Nanosafety, Division of Nanomedicine and Nanobiology, National Center for Nanoscience and Technology, China, and CAS Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety, Chinese Academy of Sciences, Beijing 100190, P.R. China

[§]Department of Chemistry & Chemical Engineering, College of Environment and Energy Engineering, Beijing University of Technology, Beijing 100124, China

[#]Molecular Imaging Laboratory, Department of Radiology, Howard University, Washington, DC 20060, USA

[&]Center for Food Safety and Applied Nutrition, Food and Drug Administration, College Park, MD 20740, USA

Abstract

Single-walled carbon nanotubes (SWCNTs) are broadly used for various biomedical applications such as drug delivery, *in vivo* imaging and cancer photothermal therapy due to their unique physiochemical properties. However, once they enter the cells, the effects of SWCNTs to the intracellular organelles and macromolecules are not comprehensively understood. Cytochrome c (Cyt c), as a key component of the electron transport chain in mitochondria, plays an essential role in cellular energy consumption, growth and differentiation. In this study, we found the mitochondrial membrane potential (MMP) and mitochondrial oxygen uptake were greatly decreased in human epithelial KB cells treated with SWCNTs, which accompanies the reduction of Cyt c. SWCNTs deoxidized Cyt c in a pH dependent manner as evidenced by the appearance of a 550 nm characteristic absorption peak, which intensity increased as pH increase. Circular dichroism measurement confirmed the pH-dependent conformational change, which facilitated closer association of SWCNTs with the heme pocket of Cyt c and thus expedited the reduction of Cyt c. The electron transfer of Cyt c is also disturbed by SWCNTs, as measured with electron spin resonance spectroscopy. In conclusion, the redox activity of Cyt c was affected by SWCNTs treatment due to attenuated electron transfer and conformational change of Cyt c, which consequently changed mitochondrial respiration of SWCNTs treated cells. This work is significant to SWCNTs research because it provided novel understanding to the disruption of SWCNTs to the mitochondria and has important implications for biomedical applications of SWCNTs.

*Address correspondence to jhsun@bjut.edu.cn or liangxj@nanoctr.cn.

[‡]These authors contributed equally to this work.

Supporting Information Available: Stability of carboxy-SWCNTs in physiological buffer and media; effect of SWCNTs and sodium cholate on the viability of KB cells; fluorescence microscopy images of ROS generation in SWCNTs-treated KB cells; quantification of Cyt c absorbed onto SWCNTs; the UV-visible spectra of Cyt c in the absence and presence of ascorbic acid; the UV-visible spectra of Cyt c interacted with SWCNTs in the presence of different concentration of sodium cholate; the UV-visible spectra of Cyt c interacted with SWCNTs at pH 7.0 with varied PB concentration; and the UV-vis spectra of Cyt c under interaction with SWCNTs in complete DMEM media. These materials are available free of charge *via* the Internet at <http://pubs.acs.org>.

Keywords

Single-walled carbon nanotubes; Carboxy; Cytochrome C; Electron transfer; Mitochondrial function; Redox activity

Carbon nanomaterials with tunable surface properties and good biocompatibility have great potential for biological and medical applications.^{1, 2} As a unique quasi one-dimensional material, SWCNTs have been explored as novel delivery vehicles for drugs, peptides, proteins, plasmid DNA and small interfering RNA.³ The interactions between biological macromolecules and carbon nanomaterials have been reported to cause changes in the structure and function of proteins.^{2, 4–10} When the functionalized carbon nanotubes recognize and bind to the catalytic site of α -chymotrypsin, its enzymatic activity was inhibited completely.⁶ Karajanagi *et al.* demonstrated that activities of α -chymotrypsin (CT) and soybean peroxidase (SBP) were differently affected by their interaction with SWCNTs. It was found that SBP retained its native three dimensional conformation and up to 30 % of its native activity after interaction with SWCNTs. In contrast, α -CT unfolded on the SWCNTs surface and retained only 1 % of its original activity.⁵ Proteins can bind with SWCNTs through covalent or non-covalent interactions, due to their large surface areas. SWCNTs tend to strongly bind proteins through charge complementarities, π - π stacking and other nonspecific interactions.^{11–13} Carboxylated-SWCNTs might interact with proteins located on the cell membrane or in the cytoplasm of multipotent mesenchymal stem cells, which have a further impact on subsequent cellular signaling pathways.¹⁴ Recent studies have shown that bacterial membrane rhodopsin proteins (bR) can be adsorbed onto SWCNTs through hydrophobic interactions between the bR α -helices and the sidewall of the nanotube. This noncovalent functionalization caused bR proteins to undergo significant secondary structural changes.¹⁵ These results suggest that an understanding of the effects of SWCNTs on protein conformation and function may be essential for developing novel biological and medical applications for SWCNTs.

Cytochrome-*c* (Cyt *c*) is a key component of the mitochondrial respiratory chain that exists in the cytosol between the inner and outer membranes of mitochondria. The heme group of Cyt *c* allows it to undergo redox reactions. Cyt *c* plays an important role in the biological respiratory chain, whose function is to receive electrons from cytochrome *c* reductase and deliver them to cytochrome *c* oxidase. The activity of Cyt *c* is essential for mitochondrial function and is further associated with a variety of processes including cellular differentiation, apoptosis, control of the cell cycle and cell growth.¹⁶ Mitochondrial dysfunction leads to cell apoptosis and is closely linked with several diseases.^{17, 18} Cyt *c* is also involved in initiation of apoptosis by abnormal accumulation in cytoplasm which initiates signal pathways leading cells to dysfunction.^{19, 20} The release of Cyt *c* from mitochondria into cytoplasm has been shown to induce or activate apoptosis through interactions with cytoplasmic proteins such as apaf-1, Bcl-2, TIB, and caspase.²¹ In addition, dysfunction of Cyt *c* can modulate the mitochondrial membrane potential (MMP) and cause mitochondrial respiratory chain malfunction.²² SWCNTs have been demonstrated to have a good promotion toward the reduction/oxidation of cytochrome *c*.²³ In another study, SWCNTs were used as transporters to shuttle Cyt *c* through the plasma membrane and into cytoplasm to regulate the activities of cells.²⁴

SWCNTs were found to localize exclusively in mitochondria of both tumor and normal cells.²⁵ To date reports on whether SWCNTs itself could affect the function and activity of Cyt *c* and the resulting effects on cellular functions are limited. Technically, it is very difficult to avoid aggregation of pristine SWCNTs when they are suspended in physiologically relevant solvents. This problem has hindered the progress of our

understanding of the interactions between Cyt c and SWCNTs. In this study, we modified SWCNTs with carboxy groups to avoid its aggregation. The redox properties of Cyt c were examined following incubation it with suspensions of SWCNTs in physiological buffer. For the first time, we demonstrate that the redox potential of Cyt c is altered by interacting with SWCNTs. In addition, the SWCNTs treatment caused MMP change and mitochondrial respiration inhibition in KB cells was measured. The results provide evidence that SWCNTs can cause MMP change and decrease the level of mitochondrial oxygen uptake in cells, which are the results of the redox activity change and impaired electron transfer of Cyt c. While the exact mechanism needs to be further explored, our studies of the interactions between SWCNTs and Cyt c provide some clues about how SWCNTs may interact with Cyt c and affect cellular functions.

RESULTS AND DISCUSSION

SWCNTs have been widely used to shuttle biomolecules including proteins, DNA and siRNA into cells.^{26–28} Whether the structure and function of these “passenger” molecules as well as cellular components can be affected by SWCNTs, and how SWCNTs affect cellular activities needs to be addressed. Because pristine SWCNTs have low solubility in water, we modified SWCNTs with carboxy groups and dispersed them in PB containing 0.5 wt% sodium cholate. The derived suspensions of individual SWCNTs have enhanced stability (Figure 1a, b). While pristine SWCNTs rapidly aggregated and precipitated out, carboxy-SWCNTs exhibited high suspension stability. We also tested the stability of carboxy-SWCNTs in complete DMEM media. As is shown in Figure S1, carboxy-SWCNTs exhibited high suspension stability in complete DMEM media likewise. These stable suspensions of SWCNTs were used in studies on Cyt c to clarify how SWCNTs affect cellular activities and interact with Cyt c.

Cellular Uptake of SWCNTs and Mitochondrial Morphology Change

In order to monitor the entry of SWCNTs into cells under selected conditions, we employed the micro-Raman technique of the G-band of SWCNTs in KB cells with an approximately 2-mm spatial resolution. In Raman mapping images, the color change represents the content of carbon nanotubes in the cells. With the increase of SWCNTs content, the color of Raman mapping images changes from black to orange. Figure 1c and d show the Raman mapping images of KB cells incubated with or without 60 $\mu\text{g/ml}$ SWCNTs for 24 h. As expected, the strong Raman signals was observed at about 1580 cm^{-1} in KB cells after incubation with SWCNTs. In contrast, almost no obvious Raman signals were observed for control cells. The presence of SWCNTs is seen as an orange area in the cells by Raman intensity ratio map. Raman mapping clearly indicates that SWCNTs entered and accumulated in KB cells with intensive near-infrared excited Raman signals.

It is known that SWCNTs exhibit a strong resonance Raman shift at about 1580 cm^{-1} , a specific characteristic graphitic stretching mode, which can be used to measure the quantity of SWCNTs. Therefore, we further examined the G band intensity of 1580 cm^{-1} in the isolated mitochondria of KB cells treated with SWCNTs from 0 to 60 $\mu\text{g/ml}$ for 24 h. It was found that the mitochondrial targeting of SWCNTs is dose-dependent, with higher Raman intensities observed for SWCNTs signals in the mitochondria isolated from cells treated with higher SWCNTs concentration (Figure 2A). To verify the entering of SWCNTs to the mitochondria, we carried out transmission electron microscopy to observe the mitochondria in KB cells treated with 60 $\mu\text{g/ml}$ of SWCNTs for 24 h. TEM images show that SWCNTs appeared in mitochondria (Figure 2B arrow c), and the mitochondrial structure was greatly affected. The mitochondria became swollen and round, with the cristae shown irregular and disordered. Some mitochondria became vacuolus and the cristae were nearly completely

destroyed or disappeared (Figure 2B). The morphology of mitochondria of KB cells treated with 20 $\mu\text{g/ml}$, 40 $\mu\text{g/ml}$, and 80 $\mu\text{g/ml}$ of SWCNTs for 24 h is shown in Figure S2.

SWCNTs Treatment Decreases the Level of Mitochondrial Oxygen Uptake

To measure whether SWCNTs can affect mitochondrial respiration, oxygen uptake was measured in KB cells in culture using a sensitive self-referencing oxygen electrode.²⁹ The oxygen consumption measurement is based on the translational movement of an oxygen-selective microelectrode at a known frequency through the gradient of the oxygen concentration around the cell. The differential current of the electrode was converted into a directional measurement of oxygen flux using the Fick equation. Mitochondria require oxygen to produce ATP in sufficient quantities to drive energy-requiring reactions in eukaryotic organisms. The oxygen flux detected reliably correlates with the metabolic state of the cell. Normal mitochondria have high oxygen flux and lower level of oxygen flux is a result of malfunctioned mitochondria. As is shown in Figure 3c and d, oxygen consumption in KB cells declined after SWCNTs treatment in a time-dependent manner. After 24 h of SWCNTs treatment, the oxygen flux of cells is only about 9.7% of that in the control group. The result clearly shows that mitochondrial oxygen consumption of cells is significantly decreased by SWCNTs treatment.

Effects of SWCNTs on Mitochondrial Membrane Potential

Mitochondria, one of the most important organelles in eukaryotic cells, furnish cellular energy through respiration and regulate cellular metabolism as a cellular power plant to maintain the growth, differentiation and proliferation of cells. The effect of SWCNTs on mitochondrial function was investigated by examining their effects on the MMP, an indicator of mitochondrial activity. We used the MTT assay to evaluate the effect of different concentrations of SWCNTs on cell viability. Our results show that the viability of KB cells decreased after exposure to SWCNTs dispersion for 24 h at high concentrations, while the viability was not significantly affected by PB buffer containing sodium cholate with the same concentration (Figure S3). Sodium cholate is a surfactant of low biological toxicity broadly used to disperse SWCNTs.³⁰ Cells treated with 60 $\mu\text{g/ml}$ SWCNTs, which would result in 70 % cell viability after 24 h of exposure, were used to investigate the effects of SWCNTs to mitochondria.

The effect of SWCNTs on the MMP of KB cells was firstly measured by flow cytometry with JC-1 staining. JC-1 existed either as a green fluorescent monomer at depolarized membrane potentials or as a red fluorescent J-aggregate at hyperpolarized membrane potentials. Changed MMPs were indicated by a fluorescence emission shift from J-aggregates with red fluorescence (~ 590 nm) to J-monomers with green fluorescence (~ 529 nm). The higher mitochondrial membrane potentials were defined with the higher ratio of red fluorescent intensity and green fluorescent intensity.³¹ Flow cytometry results are expressed as decrease or increase of fluorescence intensity, indicating the change of MMP among cell populations (Figure 4). Cells incubated with 60 $\mu\text{g/ml}$ SWCNTs showed a decrease of the red fluorescence intensity in a time-dependent manner. Carbonyl cyanide 3-chlorophenyl hydrazone (CCCP), an uncoupled agent that depolarizes mitochondrial membranes, was used as a positive control. CCCP treated group showed a dramatic decrease of red fluorescence intensity (Figure 4b). Cells in the R_2 region were cells having normal MMP while those in the R_3 region were cells having reduced MMP. The percentages appearing in each panel of Figure 4 are the percentages of cells in the R_2 and R_3 regions. The results show that, the longer KB cells are incubated with SWCNTs, the more cells exhibit a depolarized MMP. When cells were incubated with SWCNTs for 24 h, the MMP of 94.75 % cells was depolarized (Figure 4f).

To confirm the results obtained from flow cytometry, we examined the change of fluorescence in mitochondria by confocal microscopy. The MMP of KB cells in the presence or absence of SWCNTs was measured by confocal microscopy after staining with JC-1 (Figure 5). Control cells had both red and green fluorescence, co-existing in the same cell (Figure 5, top panels). Superimposition of the green and red fluorescence images revealed a large degree of overlap and displayed an orange color. Treatment with the positive control, CCCP, dramatically decreased the red fluorescence (Figure 5, middle panels) and caused a visible increase of the green fluorescence intensity. The shift in membrane potential was observed by disappearance of fluorescent red-stained mitochondria and an increase in fluorescent green-stained mitochondria. Following treatment with SWCNTs, formation of red fluorescent J-aggregates dropped, and the ratio of red fluorescence intensity to green fluorescence intensity decreased gradually, (Figure 5, bottom panels) indicating increased depolarization of mitochondria with prolonged exposure to SWCNTs. These results are consistent with the results obtained using flow cytometry.

SWCNTs Treatment Increases the ROS Level in Cells

It has been reported that Cyt c plays an important role in ROS clearance.^{32, 33} Intracellular ROS level of cells treated with SWCNTs was measured after treating the KB cells with 60 $\mu\text{g/ml}$ SWCNTs for 24 h. As is shown in Figure S4, compared with the control group, enhanced ROS level was detected in SWCNTs-treated cells, which is a direct evidence of mitochondrial electron transport disruption and blocked respiratory chain activity.

The Interaction of SWCNTs with Cyt C

Normal mitochondrial function requires the balanced interaction between many critical mitochondrial proteins. Minor changes of these proteins could result in dramatic effects on cellular metabolism. Cyt c plays an important role in mitochondrial electron transport and cell metabolism. Its role in maintaining a normal MMP is well established.³⁴ Cyt c is a well-characterized small, heme-containing redox protein with a molecular weight of 12.384 g/mol. The heme edge of Cyt c is surrounded by an array of lysine and arginine residues. Cyt c transfers electrons by undergoing oxidation and reduction of the iron cation (which can be alternately 2+ or 3+) in Cyt c's heme moiety. Oxidized Cyt c has absorption peaks at 408 nm and 530 nm, while reduced Cyt c has three peaks at 415 nm, 520 nm, and 550 nm.³⁵ The additional peak at 550 nm can, therefore, be monitored spectroscopically to quantitate the amount of reduced Cyt c. We determined that Cyt c could bind onto SWCNTs (Figure S5). The extent of binding depended on both the concentrations of Cyt c and SWCNTs. The amount of Cyt c binding with SWCNTs was quantified with UV-visible spectra at 408 nm. When the concentration of Cyt c was changed, the amount of Cyt c bound to 100 $\mu\text{g/ml}$ SWCNTs showed a linear increase and reached maximum at 150 $\mu\text{g/ml}$ (Figure S5). The adsorption of Cyt c onto SWCNTs is shown in Figure S6.

Systematic spectroscopic studies were conducted to explore the interaction between Cyt c and SWCNTs. Firstly, it was found that a new absorption peak at 550 nm, the characteristic peak of reduced Cyt c, appeared in the presence of SWCNTs (Figure 6a), similar to the positive control of ascorbic acid, which reduces Cyt c rapidly and thoroughly (Figure S7). This result clearly demonstrates that Cyt c undergoes reduction in the presence of SWCNTs. Next, the effect of pH on the interaction between SWCNTs and Cyt c was investigated. Solution with Cyt c was used as control, reduction of Cyt c was facilitated when the pH increased from 7.0 to 9.0, consistent with an increase in the absorbance at 550 nm (Figure 6b). In order to prove this phenomenon was caused only by SWCNTs, the effects of sodium cholate and ionic strength, the other two components in this system, on Cyt c reduction were evaluated at pH 7.0. There was no peak at 550 nm when the concentration of sodium cholate increased from 0.25 wt% to 2.0 wt%, indicating that sodium cholate had little effect on the

reduction (Figure S8a). The effect of PB on the interaction between SWCNTs and Cyt c was also investigated. The change of PB concentration from 10 mM to 50 mM had no significant effect on the interaction of SWCNTs with Cyt c (Figure S8b). Taken together, these results demonstrate that the reduction of Cyt c is caused only by SWCNTs and that this reduction is enhanced by an increase of pH. To find out whether the nonspecific adsorption of serum proteins onto SWCNTs surface will affect their interaction with Cyt c, we also carried out the experiment in complete DMEM media. It was found that Cyt c can still be reduced by SWCNTs in complete DMEM media (Figure S9).

Mechanism of the Interaction of SWCNTs with Cyt C

To analyze the underlying mechanism of the interaction between Cyt c and SWCNTs, we further examined the structural change of Cyt c at different pH. It has been reported that the structure of Cyt c changes in alkaline solutions. This conformational change is thought to increase the exposure of Cyt c's heme group to the solvent.^{36, 37} In this study, the reduction of Cyt c by SWCNTs was enhanced under alkaline conditions. This observation raises the possibility that, at alkaline pH, SWCNTs can more easily access the heme group of Cyt c, resulting in facilitated reduction of Cyt c.

To test this hypothesis, the effect of pH on the conformation change of Cyt c was measured using circular dichroism.³⁸ We found that the α -helical content of Cyt c decreased from 29.9 % to 25.8 % (pH 7.0 and pH 8.0, respectively) and to 23.1 % (pH 9.0) when the pH was raised (Figure 6c). The distal loop region moved away from the heme moiety at alkaline pH to form a channel with heme group.³⁶ As pH increases, the heme group of Cyt c was exposed to the solvent to a greater extent, which makes SWCNTs more accessible to the heme group and thereby increasing the percentage of Cyt c being reduced. The structure change of Cyt c made a channel to the heme pocket opened through α -helix, and allowed SWCNTs an easier access to heme group through this channel at higher pH. In addition to the CD measurement, the redox potential of Cyt c was determined using cyclic voltammetry (CV). The results of the CV measurements indicated that Cyt c had a pair of redox peaks and its redox potential was significantly enhanced in the presence of SWCNTs (Figure 6d). Based on CD results and CV results, the structure of Cyt c was changed, which increased the exposure of the heme moiety to the solvent, and then the reduction activity became more obvious in the presence of SWCNTs.

To better understand the effects of SWCNTs on the electron transfer of Cyt c, the ability of Cyt c to catalyze the oxidation of DMPO to DMPOX was studied using the Electron Spin Resonance (ESR) technique. In the presence of Cyt c, H_2O_2 caused the oxidation of DMPO to DMPOX, which gave the typical 7-line ESR spectrum shown as insert in Figure 7, the intensity of second line in the ESR spectrum of DMPOX was used to monitor the time dependence. Ascorbic acid, as a positive control, inhibited this oxidation in a concentration dependent manner (Figure 7a). This inhibition was observed as a delay in the appearance of the ESR signal for DMPOX. Similarly, addition of SWCNTs also resulted in a similar concentration dependent inhibition of oxidation (Figure 7b). As SWCNTs concentration increased, the phenomenon of the ESR signal delay for DMPOX became more obvious with the appearance time from about 150 to 350 seconds and lowered intensity from 10.5 to 4.5 a.u., indicating the slower and smaller amount of electron being transferred. These results indicated that SWCNTs can affect the electron transfer and catalysis ability of Cyt c required for the oxidation of DMPO to DMPOX.

Mitochondria is the powerhouse of the living cell, generating energy for various cellular activities. The function and integrity of mitochondria may impact the viability, proliferation/division, hypoxic tolerance of the cell and mitochondrial dysfunction is implicated in the pathogenesis of many diseases. For this reason, the study of mitochondrial function has

become central to a wide variety of clinical and basic science research. The majority of ATP generation is dependent on mitochondrial electron transport. Cyt c, as an integral mitochondrial protein, plays an important role in electron transport and cell respiration.³⁴ Cytochrome c oxidase is the terminal enzyme of the mitochondrial respiratory chain and is responsible for 90% of cellular oxygen consumption in mammals. In mitochondria, cytochrome c plays an essential role in the generation of MMP. This potential is essential for various functions including the production of ATP *via* oxidative phosphorylation.

To successfully apply the new generation of nanomaterials as drug carriers in the treatment of diseases, it is essential to determine their pharmacological and toxicological profiles. SWCNTs are commonly used as carrier to forward different payloads into cells. However, as the basis of biomedical applications of SWCNTs, their interactions with intracellular molecules in cells have not been fully understood. The potential biomedical applications of nanotubes require a better understanding of their cellular dynamics for its better usage in clinic. The toxicity of carbon nanotubes has been an important issue in carbon nanomaterial research. The potential cytotoxicity includes metabolic pathway disruption, redox regulation, cytoskeleton formation alteration, and cell growth inhibition, *etc.* Yuan *et al.* found two cytoskeleton related proteins, profilin and filamin, were upregulated by approximately 35% in oxidized SWCNTs treated cells. The upregulation of these cytoskeleton proteins suggested that the exposure of SWCNTs might alter the intracellular microfilament network.³⁹ Kagan *et al.* demonstrated that iron-rich SWCNT caused a significant loss of intracellular low molecular weight thiols (GSH) and accumulation of lipid hydroperoxides in murine macrophages.⁴⁰ It has been reported that mitochondria is the target organelle for the cytotoxicity of SWCNTs.⁴¹ Consistent with the reports of other investigators, we have found that treatment of cells with SWCNTs resulted in accumulation of the SWCNTs in cells. At low concentrations, for short period, SWCNTs inhibits cytochrome c oxidase to a lower extent, but higher period of SWCNTs exposure results in inhibition of respiration, leading to the development of “metabolic hypoxia” – a situation in which, although oxygen is available, the cell is unable to utilize it. We have further determined that this accumulation of SWCNTs was accompanied by a reduction in the MMP. Cytochrome c molecules can be successfully absorbed non-covalently on carbon nanotubes.⁴² The interaction most likely occurs *via* the protein side that is rich in Lys residues, since the primary amines strongly bind to carboxylate functionalities, and amines are also known to strongly interact with the carbon-nanotube sidewall.⁴³ The effect of SWCNTs on the electron-transfer reaction of cytochrome c has been previously studied in biosensor research. Wang *et al.* has found the activated SWCNT-modified electrode has good promotion toward the reduction/oxidation of cytochrome c.²³ Our results suggest that SWCNTs may directly interact with Cyt c to alter its oxidation state and redox potential, resulting in an alteration in Cyt c’s ability to facilitate electron transport. Disruption in electron transfer has many unfavorable consequences, it has been reported that disrupting electron transfer at cytochrome c oxidase may result in ionic readjustment and modulation of the Ca²⁺ flux between the mitochondria and the ER.⁴⁴

CONCLUSIONS

In this study, we investigated the effect of SWCNTs on mitochondria function and the interactions of SWCNTs with Cyt c, an important component of the electron transport chain in mitochondria. Based on the extracellular interactions of cytochrome c with carbon nanotubes, and the intracellular mitochondrial dysfunction induced by SWCNTs treatment, we primary conclude that the effects of SWCNTs on mitochondria should be a stepwise process: (1) SWCNTs enter the cells and localize in the mitochondria (2) Cyt c is partially reduced when interacting with SWCNTs and the redox activity of Cyt c is affected (3) The electron transfer of Cyt c is decreased and attenuated (4) MMP is decreased (5)

Mitochondrial respiratory chain is disrupted and mitochondrial oxygen uptake is greatly reduced (6) Metabolic hypoxia is caused by SWCNTs and the mitochondrial electron transport-dependent ATP generation is affected. Identification of pharmacological and toxicological profiles is of critical importance for the use of nanoparticles as drug carriers in nanomedicine and for the biosafety evaluation of environmental nanoparticles in nanotoxicology. The findings of this study will broaden and deepen our understanding of the interaction of SWCNTs with Cyt C and the mechanism of mitochondria dysfunction caused by SWCNTs. When using SWCNTs to fulfill different applications such as live imaging, photothermal therapy and pharmaceutical carriers in the future, its disruption to the mitochondria function and cellular respiration should be considered.

METHODS

Materials

Pristine SWCNTs were purchased from Chengdu Organic Chemicals Company Ltd. of Chinese Academy of Sciences. Carbonyl cyanide 3-chlorophenyl hydrazone (CCCP) and sodium cholate were obtained from Alfa Aesar (Ward Hill, MA). Oxidized Cyt c from horse heart, the spin trap, 5, 5-dimethyl N-oxide pyrroline (DMPO), and L-ascorbic acid were purchased from Sigma (St. Louis, MO). Mitochondria were isolated by a Mitochondria Isolation Kit (Pierce, Rockford, IL) for Cultured Cells. All chemicals were of analytical grade and were used without further purification.

Preparation of Carboxyl-SWCNTs

In order to improve their dispersity and stability in solutions, pristine SWCNTs were treated by acid oxidation. Briefly, 0.1g pristine SWCNTs and 100 ml of 3:1 (v/v) mixture of 98 % H_2SO_4 and 68 % HNO_3 were mixed and sonicated for 2 h. The resulting dark suspension was cooled to room temperature and diluted with distilled water. The diluted suspension was filtered through a 0.22 μm polycarbonate membrane (Millipore, USA), rinsed with distilled water until the pH was greater than 5. Finally, the purified carboxy-SWCNTs were dried at room temperature. Carboxyl-SWCNTs were suspended in phosphate buffer (30 mM, pH 7.0) with 0.5 wt% sodium cholate surfactant and sonicated for 2 h. The suspension was then centrifuged at 1500 rpm for 10 min to remove large bundles. The concentration of carboxyl-SWCNTs was determined by UV- visible spectroscopy.

MTT Assay

The cell viability of human epithelial carcinoma cells (KB cells, kindly provided by Dr. Michael M. Gottesman, CCR, NCI) treated with SWCNTs was determined using the MTT assay. Cells were stained with 100 μl of sterilized MTT dye ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 0.5 mg/mL, Sigma-Aldrich) for 4 h at 37 °C, and 100 μl of DMSO was added. The spectrometric absorbance at 570 nm was measured on a microplate reader (M200, Tecan, Mannedorf, Switzerland).

Micro-Raman Mapping

After 24 h of treatment with SWCNTs, KB cells were washed twice to remove free SWCNTs and the media was replaced with PBS. The scanning area was selected by focusing on the cells using the microscope, and the micro-Raman laser was then focused on the dish (λ excitation=785nm) using the Renishaw Micro-Raman Spectroscopy System (Renishaw plc, Wotton-under-Edge, UK). A line scanning mode was applied to get a Raman spectrum at every point in the selected area (1 $\mu\text{m} \times 1 \mu\text{m}$ for each point). The image was obtained by plotting the integrated area in the range of 1580–1610 cm^{-1} of the Raman spectra obtained in the cell-containing area.

Oxygen Flux Measurements

Oxygen consumption was recorded according to the self-referencing method^{29, 45}. Briefly, a platinum electrode was placed 5 μm from the cell, and then moved rapidly back and forth over a 10 μm distance between two points located 5 and 15 μm away from the cell soma in the X–Y axes, so that the oxygen-sensing electrode was positioned repeatedly closer to and farther from the cell. This amperometric probe was polarized with -0.6V , at which voltage the reduction of oxygen at the probe tip was diffusion-limiting, and therefore $[\text{O}_2]$ in the solution could be assessed. As the O_2 concentration near the cell is smaller than in the far position, the difference in current between the near and the far position of the platinum electrode represents the gradient of the oxygen concentration around the cell, a reflection of O_2 consumption. We then converted the difference in current into oxygen flux. The oxygen flux level was measured every 4.73s. Figure 3c is the representative trace of the oxygen flux level of one single cell under each treatment. Figure 3d is the average oxygen flux value of all the measured values of all the cells under each treatment.

Flow Cytometry Measurements

The MMP was measured with 1.5 μM JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) (Invitrogen, Rockville, MD) by flow cytometry. KB cells were seeded in 6-well plates and incubated for 24 h. Then cells were incubated in fresh DMEM media or in media containing SWCNTs for 4 h, 12 h, 18 h, and 24 h. As a known MMP disrupter, 100 μM carbonyl cyanide 3-chlorophenyl hydrazone (CCCP, Alfa Aesar, Haverhill, MA) was used as positive control. After incubation, cells were trypsinized, collected by centrifugation, and then incubated with medium containing JC-1 dye for 20 min at 37 $^{\circ}\text{C}$. Finally, the cells were washed and resuspended in 1 ml of PBS for fluorescent analysis using a FACScan flow cytometer. J-aggregate fluorescence was recorded by flow cytometry in fluorescence channel 2 (FL2) and monomer fluorescence in fluorescence channel 1 (FL1).

Confocal Microscopy Imaging

KB cells were seeded in polylysine-coated glass bottom dishes. After 24 h, the cells were treated with SWCNTs for 4 h, 12 h, 18 h, and 24 h. The cells were then stained with JC-1 dye as previously described for flow cytometry and examined by confocal microscopy (Ultraview VOX, Perkin-Elmer, Waltham, MA).

Measurement of Interactions between Cyt C and Carboxyl-SWCNTs

Cyt c was incubated with selected concentrations of carboxyl-SWCNTs for 1 h at room temperature. After incubation, the suspension was centrifuged at 13000 rpm for 30 min and the supernatant was removed. The amount of Cyt c in the supernatant was measured using UV-visible spectroscopy (Lambda 950, Perkin-Elmer, USA). The UV absorption peak at 408nm, attributable to the heme group in the Soret region of the Cyt c spectrum, was used to quantitate Cyt c.

UV-visible Spectroscopy Assay

The reduction of oxidized Cyt c in the presence of SWCNTs was measured using the absorption of Cyt c at 550nm characteristic for reduced Cyt c. Ascorbic acid, a known reducing agent, was used as positive control. Briefly, oxidized Cyt c was dissolved in PB solution. The spectra were measured after SWCNTs (0.1 mg/ml) or ascorbic acid (5 mg/ml) was added. In addition, to measure the effect of the surfactant, sodium cholate, on the reduction of Cyt c by SWCNTs, UV-visible absorption spectra were obtained for solutions containing Cyt c (150 $\mu\text{g}/\text{ml}$), SWCNTs (100 $\mu\text{g}/\text{ml}$) and sodium cholate ranging from 0.25 to 2 wt%. To study the effect of pH on the interaction between SWCNTs and Cyt c,

SWCNTs (100 $\mu\text{g/ml}$) were incubated with oxidized Cyt c (150 $\mu\text{g/ml}$) in PB with increasing pH from 7.0 to 9.0.

Circular Dichroism (CD) Measurement

CD spectra in the far-UV (200–250nm), near-UV (250–350nm), as well as the Soret (350–450nm) spectral regions were recorded using a spectropolarimeter (J-720, Jasco). Spectra were obtained for samples containing 600 $\mu\text{g/ml}$ oxidized Cyt c in the absence or presence of SWCNTs (400 $\mu\text{g/ml}$) at different pH. CD spectra were measured using quartz cells with 1 mm path length. All spectra were corrected for background absorption. Each experiment was repeated three times with a bandwidth of 1.0 nm and scanning rate of 100 nm/min. All the measurements were performed at a constant temperature of 20°C.

Cyclic Voltammetry Analysis

A three-electrode cell with Indium Tin Oxides (ITO) as working electrode, Pt as counter electrode, and $\text{Hg/Hg}_2\text{Cl}_2$ as reference electrode was used for the measurements by Potentiostat with PHE200TM Physical Electrochemistry analysis (Gamry Instruments, Warminster, PA). The working electrode area is identical among samples. The cyclic voltammograms were recorded immediately after inserting the reference electrode into the Potentiostat analyte solution (Princeton Applied Research, Oak Ridge, TN). The potential cycling was started at 0.3 V and allowed to proceed till -0.1 V, and the cycle was completed by stopping the scan at 0.3 V (scan rate 50 mV/s).

Electron Spin Resonance (ESR) Measurements

Oxidation of the spin trap DMPO, to form 5,5-dimethyl-1-pyrrolidone-N-oxyl (DMPOX), was monitored by ESR to determine the effects of SWCNTs on Cyt c's ability to catalyze oxidation of substrates by H_2O_2 . Conventional ESR spectra were obtained using a Bruker EMX ESR Spectrometer (Billerica, MA). ESR spectral measurements were obtained using the following settings: 10 mW microwave power, 1 G field modulation and 100 G scan range. The time dependence of the ESR signal for solutions containing 50 mM DMPO, 0.1 mM Cyt c and 0.5 mM H_2O_2 with or without ascorbic acid (from 0.01 to 0.1 mM) was measured. Similarly, the time dependence of the ESR signal with 10 $\mu\text{g/ml}$ SWCNTs (from 2.5 to 7.5 μl), substituted for ascorbic acid, was measured. The time dependence of the ESR signal intensity was obtained at a fixed field position (the peak position of the center line of ESR spectrum). Data collection began 1 min after sample mixing. For measurement of the time dependence of the ESR signal, a scan time of 30 minutes at the described fixed field position was used. All measurements were performed in triplicates at ambient temperature.

ROS Levels Detection

The cells treated with or without SWCNTs for 24 h were stained for 30 min with 10 μM ROS probe DCFH-DA (Molecular Probes, Eugene, Oregon, USA) in PBS solution with 0.4% glucose. After the free probes were washed, the cells were observed using fluorescence microscopy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the Chinese Natural Science Foundation project (No.30970784 and 81171455), National Key Basic Research Program of China (2009CB930200), Chinese Academy of Sciences (CAS) "Hundred Talents Program" (07165111ZX) and CAS Knowledge Innovation Program. This work was

supported in part by NIH/NCRR 3 G12 RR003048, NIH/NIMHD 8 G12 MD007597, and USAMRMC W81XWH-10-1-0767 grants. This article is not an official U.S. Food and Drug Administration (FDA) guidance or policy statement. No official support or endorsement by the U.S. FDA is intended or should be inferred. This work was supported by a regulatory science grant under the FY11 FDA Nanotechnology CORES Program (JJ Yin and W. Wamer).

REFERENCES AND NOTES

1. Liu Z, Tabakman S, Welsher K, Dai HJ. Carbon Nanotubes in Biology and Medicine: *In Vitro* and *in Vivo* Detection, Imaging and Drug Delivery. *Nano Res.* 2009; 2:85–120. [PubMed: 20174481]
2. Prato M, Kostarelos K, Bianco A. Functionalized Carbon Nanotubes in Drug Design and Discovery. *Acc Chem Res.* 2008; 41:60–68. [PubMed: 17867649]
3. Zhou F, Xing D, Wu B, Wu S, Ou Z, Chen WR. New Insights of Transmembranal Mechanism and Subcellular Localization of Noncovalently Modified Single-Walled Carbon Nanotubes. *Nano Lett.* 2010; 10:1677–1681. [PubMed: 20369892]
4. Bayraktar H, Ghosh PS, Rotello VM, Knapp MJ. Disruption of Protein-Protein Interactions Using Nanoparticles: Inhibition of Cytochrome C Peroxidase. *Chem Commun (Camb).* 2006:1390–1392. [PubMed: 16550276]
5. Karajanagi SS, Vertegel AA, Kane RS, Dordick JS. Structure and Function of Enzymes Adsorbed onto Single-Walled Carbon Nanotubes. *Langmuir.* 2004; 20:11594–11599. [PubMed: 15595788]
6. Zhang B, Xing Y, Li Z, Zhou H, Mu Q, Yan B. Functionalized Carbon Nanotubes Specifically Bind to Alpha-Chymotrypsin's Catalytic Site and Regulate its Enzymatic Function. *Nano Lett.* 2009; 9:2280–2284. [PubMed: 19408924]
7. Wijaya I, Gandhi S, Nie T, Wangoo N, Rodriguez I, Shekhawat G, Suri C, Mhaisalkar S. Protein/Carbon Nanotubes Interaction: The Effect of Carboxylic Groups on Conformational and Conductance Changes. *Appl Phys Lett.* 2009; 95:073704.
8. Yi C, Fong C, Zhang Q, Lee S, Yang M. The Structure and Function of Ribonuclease A upon Interacting with Carbon Nanotubes. *Nanotechnology.* 2008; 19:095102. [PubMed: 21817664]
9. Bayraktar H, You CC, Rotello VM, Knapp MJ. Facial Control of Nanoparticle Binding to Cytochrome C. *J Am Chem Soc.* 2007; 129:2732–2733. [PubMed: 17309259]
10. Sandanaraj B, Bayraktar H, Krishnamoorthy K, Knapp M, Thayumanavan S. Recognition and Modulation of Cytochrome C's Redox Properties Using an Amphiphilic Homopolymer. *Langmuir.* 2007; 23:3891–3897. [PubMed: 17315896]
11. Shang W, Nuffer JH, Dordick JS, Siegel RW. Unfolding of Ribonuclease A on Silica Nanoparticle Surfaces. *Nano Lett.* 2007; 7:1991–1995. [PubMed: 17559285]
12. Cedervall T, Lynch I, Lindman S, Berggard T, Thulin E, Nilsson H, Dawson KA, Linse S. Understanding the Nanoparticle-Protein Corona using Methods to Quantify Exchange Rates and Affinities of Proteins for Nanoparticles. *Proc Natl Acad Sci U S A.* 2007; 104:2050–2055. [PubMed: 17267609]
13. Yi C, Fong C, Chen W, Qi S, Tzang C, Lee S, Yang M. Interactions between Carbon Nanotubes and DNA Polymerase and Restriction Endonucleases. *Nanotechnology.* 2007; 18:025102.
14. Liu D, Yi C, Zhang D, Zhang J, Yang M. Inhibition of Proliferation and Differentiation of Mesenchymal Stem Cells by Carboxylated Carbon Nanotubes. *ACS Nano.* 2010; 4:2185–2195. [PubMed: 20218664]
15. Bertoncini P, Chauvet O. Conformational Structural Changes of Bacteriorhodopsin Adsorbed onto Single-walled Carbon Nanotubes. *J Phys Chem B.* 2010; 114:4345–4350. [PubMed: 20218568]
16. Li K, Li Y, Shelton JM, Richardson JA, Spencer E, Chen ZJ, Wang X, Williams RS. Cytochrome C Deficiency Causes Embryonic Lethality and Attenuates Stress-Induced Apoptosis. *Cell.* 2000; 101:389–399. [PubMed: 10830166]
17. Maloyan A, Sanbe A, Osinska H, Westfall M, Robinson D, Imahashi K, Murphy E, Robbins J. Mitochondrial Dysfunction and Apoptosis Underlie the Pathogenic Process in Alpha-B-Crystallin Desmin-Related Cardiomyopathy. *Circulation.* 2005; 112:3451–3461. [PubMed: 16316967]
18. Irwin WA, Bergamin N, Sabatelli P, Reggiani C, Megighian A, Merlini L, Braghetta P, Columbaro M, Volpin D, Bressan GM, et al. Mitochondrial Dysfunction and Apoptosis in Myopathic Mice with Collagen VI Deficiency. *Nat Genet.* 2003; 35:367–371. [PubMed: 14625552]

19. Chandra D, Bratton SB, Person MD, Tian Y, Martin AG, Ayres M, Fearnhead HO, Gandhi V, Tang DG. Intracellular Nucleotides Act as Critical Prosurvival Factors by Binding to Cytochrome C and Inhibiting Apoptosome. *Cell*. 2006; 125:1333–1346. [PubMed: 16814719]
20. Hao Z, Duncan GS, Chang CC, Elia A, Fang M, Wakeham A, Okada H, Calzascia T, Jang Y, You-Ten A, et al. Specific Ablation of the Apoptotic Functions of Cytochrome C Reveals a Differential Requirement for Cytochrome C and Apaf-1 in Apoptosis. *Cell*. 2005; 121:579–591. [PubMed: 15907471]
21. Zhivotovsky B, Orrenius S, Brustugun O, Dskeland S. Injected Cytochrome C Induces Apoptosis. *Nature*. 1998; 391:449–450. [PubMed: 9461210]
22. Waterhouse NJ, Goldstein JC, von Ahsen O, Schuler M, Newmeyer DD, Green DR. Cytochrome C Maintains Mitochondrial Transmembrane Potential and ATP Generation after Outer Mitochondrial Membrane Permeabilization during the Apoptotic Process. *J Cell Biol*. 2001; 153:319–328. [PubMed: 11309413]
23. Wang J, Li M, Shi Z, Li N, Gu Z. Direct Electrochemistry of Cytochrome C at a Glassy Carbon Electrode Modified with Single-Wall Carbon Nanotubes. *Anal Chem*. 2002; 74:1993–1997. [PubMed: 12033297]
24. Kam NW, Dai H. Carbon Nanotubes as Intracellular Protein Transporters: Generality and Biological Functionality. *J Am Chem Soc*. 2005; 127:6021–6026. [PubMed: 15839702]
25. Zhou F, Wu S, Wu B, Chen WR, Xing D. Mitochondria-Targeting Single-walled Carbon Nanotubes for Cancer Photothermal Therapy. *Small*. 2011; 7:2727–2735. [PubMed: 21861293]
26. Kam NW, Liu Z, Dai H. Carbon Nanotubes as Intracellular Transporters for Proteins and DNA: an Investigation of the Uptake Mechanism and Pathway. *Angew Chem Int Ed Engl*. 2006; 45:577–581. [PubMed: 16345107]
27. Liu Z, Tabakman SM, Chen Z, Dai H. Preparation of Carbon Nanotube Bioconjugates for Biomedical Applications. *Nat Protoc*. 2009; 4:1372–1382. [PubMed: 19730421]
28. Liu Z, Winters M, Holodniy M, Dai H. siRNA Delivery into Human T cells and Primary Cells with Carbon-Nanotube Transporters. *Angew Chem Int Ed Engl*. 2007; 46:2023–2027. [PubMed: 17290476]
29. Land SC, Porterfield DM, Sanger RH, Smith PJ. The Self-Referencing Oxygen-Selective Microelectrode: Detection of Transmembrane Oxygen Flux from Single Cells. *J Exp Biol*. 1999; 202:211–218. [PubMed: 9851909]
30. Blackburn JL, McDonald TJ, Metzger WK, Engtrakul C, Rumbles G, Heben MJ. Protonation Effects on the Branching Ratio in Photoexcited Single-walled Carbon Nanotube Dispersions. *Nano Lett*. 2008; 8:1047–1054. [PubMed: 18318507]
31. Mathur A, Hong Y, Kemp BK, Barrientos AA, Erusalimsky JD. Evaluation of Fluorescent Dyes for the Detection of Mitochondrial Membrane Potential Changes in Cultured Cardiomyocytes. *Cardiovasc Res*. 2000; 46:126–138. [PubMed: 10727661]
32. Atlante A, Calissano P, Bobba A, Azzariti A, Marra E, Passarella S. Cytochrome c Is Released from Mitochondria in a Reactive Oxygen Species (ROS)-Dependent Fashion and Can Operate as a ROS Scavenger and as a Respiratory Substrate in Cerebellar Neurons Undergoing Excitotoxic Death. *J Biol Chem*. 2000; 275:37159–37166. [PubMed: 10980192]
33. Skulachev VP. Cytochrome c in the Apoptotic and Antioxidant Cascades. *FEBS Lett*. 1998; 423:275–280. [PubMed: 9515723]
34. Dalmonte ME, Forte E, Genova ML, Giuffrè A, Sarti P, Lenaz G. Control of Respiration by Cytochrome C Oxidase in Intact Cells: Role of the Membrane Potential. *J Biol Chem*. 2009; 284:32331–32335. [PubMed: 19776013]
35. Margoliash E, Frohwirt N. Spectrum of Horse-Heart Cytochrome C. *Biochem J*. 1959; 71:570–572. [PubMed: 13638266]
36. Assfalg M, Bertini I, Dolfi A, Turano P, Mauk AG, Rosell FI, Gray HB. Structural Model for an Alkaline Form of Ferricytochrome C. *J Am Chem Soc*. 2003; 125:2913–2922. [PubMed: 12617658]
37. Sivakolundu S, Mabrouk P. Cytochrome C Structure and Redox Function in Mixed Solvents are Determined by the Dielectric Constant. *J Am Chem Soc*. 2000; 122:1513–1521.

38. Kerr LE, Birse-Archbold JL, Short DM, McGregor AL, Heron I, Macdonald DC, Thompson J, Carlson GJ, Kelly JS, McCulloch J, et al. Nucleophosmin is a Novel Bax Chaperone that Regulates Apoptotic Cell Death. *Oncogene*. 2007; 26:2554–2562. [PubMed: 17072349]
39. Yuan J, Gao H, Sui J, Duan H, Chen WN, Ching CB. Cytotoxicity Evaluation of Oxidized Single-Walled Carbon Nanotubes and Graphene Oxide on Human Hepatoma HepG2 cells: An iTRAQ-Coupled 2D LC-MS/MS Proteome Analysis. *Toxicol Sci*. 2012; 126:149–161. [PubMed: 22157353]
40. Kagan VE, Tyurina YY, Tyurin VA, Konduru NV, Potapovich AI, Osipov AN, Kisin ER, Schwegler-Berry D, Mercer R, Castranova V, et al. Direct and Indirect Effects of Single Walled Carbon Nanotubes on RAW 264.7 Macrophages: Role of Iron. *Toxicol Lett*. 2006; 165:88–100. [PubMed: 16527436]
41. Yang Z, Zhang Y, Yang Y, Sun L, Han D, Li H, Wang C. Pharmacological and Toxicological Target Organelles and Safe Use of Single-Walled Carbon Nanotubes as Drug Carriers in Treating Alzheimer Disease. *Nanomed Nanotechnol Biol Med*. 2010; 6:427–441.
42. Azamian BR, Davis JJ, Coleman KS, Bagshaw CB, Green MLH. Bioelectrochemical Single-Walled Carbon Nanotubes. *J Am Chem Soc*. 2002; 124:12664–12665. [PubMed: 12392405]
43. Heering HA, Williams KA, de Vries S, Dekker C. Specific Vectorial Immobilization of Oligonucleotide-Modified Yeast Cytochrome C on Carbon Nanotubes. *Chemphyschem*. 2006; 7:1705–1709. [PubMed: 16807958]
44. Xu W, Charles IG, Moncada S. Nitric oxide: Orchestrating Hypoxia Regulation through Mitochondrial Respiration and the Endoplasmic Reticulum Stress Response. *Cell Res*. 2005; 15:63–65. [PubMed: 15686630]
45. Smith, PJS.; Sanger, RH.; Messerli, MA. Principles, Development and Applications of Self-Referencing Electrochemical Microelectrodes to the Determination of Fluxes at Cell Membranes. In: Michael, AC.; Borland, LM., editors. *Electrochemical Methods for Neuroscience*. CRC Press; Boca Raton (FL): 2007. p. 373-405.

Figure 1. Characterization and cellular penetration of SWCNTs

TEM images and dispersion images of (a) pristine SWCNTs and (b) carboxy-SWCNTs. Raman mapping images of carboxy-SWCNTs penetration into KB cells. G-mode (1580 cm^{-1}) Raman intensity images of SWCNTs in single KB cell (c) incubated in PB buffer as control for 24 h, (d) incubated with 60 $\mu\text{g/ml}$ carboxyl-SWCNTs for 24 h. Inset: regular optical microscope images of KB cell. Scale is 10 $\times$ 10 μm .

Figure 2. Entering of SWCNTs to the mitochondria and mitochondria structure change
 (A) Raman spectra of isolated mitochondria of cells treated with different concentration of SWCNTs from 0 to 60 $\mu\text{g/ml}$ for 24 h. Curves represented G band (1580 cm^{-1}) Raman spectra of SWCNTs in mitochondria, extracted with mitochondria isolation kit, measured with equal quantities. (B) Morphology of mitochondria of SWCNTs treated cells examined by transmission electron microscopy. Swelling of mitochondria is indicated by arrows. Arrow a: Mild mitochondrial damage: the cristae are smaller. Arrow b: Severe mitochondrial damage. The cristae are nearly completely destroyed or disappeared. Arrow c: SWCNTs locate in a mitochondrion. Scale bar: 500 nm and 100 nm.

Figure 3. SWCNTs alters O₂ consumption by KB cells

(a) A picture shows the self-reference electrode. The system is set up on top of a spinning disk confocal microscope. (b) Photomicrograph shows a self-referencing electrode positioned next to a single KB cell. (c) Representative traces of oxygen flux levels of single KB cells. (d) Group data for basal respiration levels of single KB cells (control), or KB cells treated with 60 $\mu\text{g/ml}$ SWCNTs from 4 to 24 hours. At least three independent cultures were used for each study.

Figure 4. SWCNTs treatment significantly triggered MMP decrease

Changes of MMP in KB cells were examined by flow cytometry. (a) KB cells were cultured in DMEM media as control. (b) KB cells treated with CCCP were used as a positive control. (c–f) Cells were treated with 60 $\mu\text{g}/\text{ml}$ of SWCNTs for 4 h, 12 h, 18 h, and 24 h. The numbers indicate the percentage of KB cells with undepolarized (R2) or depolarized (R3) membrane potential.

Figure 5. Mitochondrial depolarization imaged by confocal microscopy

MMP of cells stained with JC-1 dye was measured with confocal microscopy. Left and middle images showed the fluorescence of green fluorescent J-monomer and red fluorescent J-aggregate, respectively. The right images showed the overlay of two images. The results were consistent with FACS quantitative measurements. Scale bar: 20 μ m.

Figure 6. Interaction of SWCNTs with Cyt C was indicated with different measurements
(a) The UV-vis spectra of Cyt c, SWCNTs and SWCNT + Cyt c. The absorption spectrum was magnified in the inserted graph as characteristic peak of Cyt c at 550 nm **(b)** The UV-vis spectra of Cyt c under interaction with SWCNTs at different pH from 7.0 to 9.0. The adsorption spectrum was magnified in the inserted graph as characteristic peak of Cyt c at 550 nm. **(c)** CD spectra of Cyt c at different pH from 7.0 to 9.0. **(d)** Cyclic voltammogram of Cyt c (150 $\mu\text{g/ml}$) were measured with or without SWCNTs (100 $\mu\text{g/ml}$) and 10 mM PB at pH 7.0 Scan rate = 50 mV s^{-1} .

Figure 7. Time dependence ESR signal of DMPOX measured during oxidation of the spin trap DMPO by Cyt c/H₂O₂ at ambient temperature

The intensity of the second line in the ESR spectrum of DMPOX was used to monitor the time dependence. **(a)** The time dependence of the ESR signal for solutions containing 50 mM DMPO, 0.1 mM Cyt c and 0.5 mM H₂O₂ without (Ctr: control) or with ascorbic acid (AA1: 0.01 mM ascorbic acid; AA2: 0.05 mM ascorbic acid; AA3: 0.1 mM ascorbic acid) was measured at pH 7.0. **(b)** The time dependence of the ESR signal with 10 Jg/ml SWCNTs (NT1: 2.5 μl; NT2: 5 μl; NT3: 7.5 μl), substituted for ascorbic acid, was measured at pH 7.0. The typical 7-line ESR spectrum of DMPOX was given as insert after oxidation of DMPO in the presence of Cyt c and H₂O₂