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Cytotoxicity effect assessment of acid purified carbon nanotubes modified with cetyltrimethyl ammonium bromide

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Abstract: The cytotoxicities of single-walled carbon nanotubes (SWNTs) and acid purified single-walled carbon nanotubes (SWNT-COOH) were investigated by spectroscopic analysis. Cell viability and cell apoptosis were applied to assessing the cytotoxicity of SWNT-COOH, cetyltrimethyl ammonium bromide (CTAB) and acid purified carbon nanotubes modified with cetyltrimethyl ammonium bromide (SWNT-COOH/CTAB). The results indicate that SWNTs are more toxic than SWNT-COOH. Concentration and time−curve analyses indicate that cytotoxicity of SWNT-COOH/CTAB is more related to the toxicity of the surfactant CTAB. The cytotoxicity effect of CTAB and SWNT-COOH/CTAB is acceptable at low concentrations (0.5−25 μg/mL). The cytotoxicity observation suggests that SWNT-COOH/CTAB can safely applied to biomedical field at low concentrations (0.5−25 μg/mL).

Key words: single-walled carbon nanotube; cetyltrimethyl ammonium bromide; cytotoxicity; acid purification; apoptosis

1 Introduction

The potential use of single-walled carbon nanotubes (SWNTs) in scientific research was first published in 1993 [1]. Owing to the unique optional, thermal, chemical and mechanical properties of carbon nanotubes, they gained and triggered significant interest in the engineering field [2,3]. Then they attracted great attention for various biomedical applications [4], such as imaging, biosensors, drugs, biological scaffold, gene delivery and cancer therapy [5,6]. But their cytotoxicity effect is still a controversial issue which restricts its biomedical applications [7]. There are many factors such as dispersibility, impurities, length and surfactants contribute to the cytotoxicity of CNTs [8,9]. The carbon nanotubes (CNTs) generate oxidative stress, decrease cell viability and induce apoptosis [10]. So, various methods are tried to decrease its cytotoxicity. Carbon nanotubes purified by carboxylic acid (CNT-COOH) have been widely used because they could well reduce impurities of catalysts, shorten the nanotubes and make some surfactants easier to conjugates with them. But ZHU et al [11] found that CNTs-COOH were more toxic than CNTs, as they reduced the cell viability in a dose-dependent manner. So, the cytotoxicity of acid purified single-walled carbon nanotubes (SWNTs-COOH) is still a controversial issue [12]. To date, only a few studies have compared the cytotoxicity of SWNTs and SWNTs-COOH [11,12].

Various surfactants were used to disperse carbon nanotubes [13]. Surfactants modified to CNTs could decrease its cytotoxicity and get it more easily combined with other molecules. CTAB is a cationic and non-covalent surfactant, which has been widely applied as a wally surfactant in electrode and biological biosensor [14,15]. In our previous study, it was found that CTAB has a stronger tendency to bind with nucleic acid [16], so it maybe has a bright future to be used as a surfactant conjugated to single-walled carbon nanotubes (SWNT-COOH/CTAB) for the purpose of gene transfection. ZHANG et al [17] reported that CTAB-PBCA

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(cetyltrimethyl ammonium bromide polybutylcyano-acrylate) nanoparticles were avirulent to $HepG_2$ cells. While GUO et al [18] found that modification with CTAB caused a damage to cells. So before considering its biological applications, cytotoxicity effect on it should be taken into consideration. CTAB can easily bind with negatively charged nucleic acid to make single-walled carbon nanotubes gene carrier. So it is meaningful to assess the cytotoxicity with CTAB, a cationic surfactant. In our study, the cytotoxicity and apoptosis effect of SWNTs, SWNT-COOH and SWNT-COOH/CTAB were evaluated.

2 Experimental

2.1 Preparation of SWNT conjugates

In this experiment, SWNTs (out diameter 1−2 nm, length 1−3 μm) and SWNT-COOH (out diameter 1−2 nm, length 1−3 μm) powders were purchased from Chengdu organic chemical company (Sichuan, China). CTAB powders were obtained from State Key Laboratory for Powder Metallurgy, Central South University, China.

The SWNTs, SWNTs-COOH and SWNT-COOH/CTAB solution were prepared by ultrasonic treatment and centrifugation method. First, SWNT powder, SWNT-COOH powder, mixture of SWNT-COOH and CTAB powder were dispersed in deionized water using ultrasonic tip (MODEL:CV188, China) operating at 70 W for 120 min to obtain SWNT suspension, SWNT-COOH suspension and SWNT-COOH /CTAB suspension. Dispersion of SWNTs, SWNT-COOH and SWNT-COOH/CTAB suspension were prepared at concentrations ranging from 0.5 to 100 μg/mL. In this process, concentration of the surfactant was kept constant (CTAB 0.3 mg/mL). Then, centrifugation (eppendorf centrifuge 5417R, Germany) with SWNT suspension, SWNT-COOH suspension and SWNT-COOH/CTAB suspension were respectively conducted at 13000*g* for 30 min. Then, we discarded the precipitates containing catalysts, bundles of nanotubes and amorphous carbon debris.

2.2 Characterization of water-dispersible SWNTs

SWNTs and SWNT-COOH powders were determined qualitatively by X-ray diffraction (XRD). Meanwhile, their quantitative analyses were performed through thermogravimetric analysis (TGA) and energy disperse spectroscopy (EDS). Thermogravimetric analysis was performed on SWNTs and SWNTs-COOH. The heating rate was 10 °C/min. The temperature was increased from room temperature to 700 °C in the presence of normal atmosphere.

2.3 Cell culture

HeLa cells obtained from Institute of Biochemistry and Cell biology(China) were used for evaluating toxicity. Cells were grown in a humidified incubator at 37 °C (5%CO_2) using DMEM medium (Gibco), which was supplemented with 10% fetal bovine serum (Gibco).

2.4 Analysis of cell viability

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay(Sigma) was used to determine cell viability. The metabolic activity of cells is proportional to the color density formed. The cells viability was calculated through the formula $(OD_e/OD_c)\times100\%$, OD_e represents the absorbance value of experimental group, and OD_c represents the absorbance value of control group).

HeLa cells were grown in 96 well microplates for 12 h. These cells were washed with phosphate buffered saline (PBS), and the medium was changed to 10% fetal bovine serum (FBS). Then, these cells were individually exposed to SWNT-COOH, surfactant CTAB, and CTAB coated SWNT-COOH suspensions, with the concentration varying in the range of 0.5−100 μg/mL for 48 h. For another group, CTAB coated SWNT-COOH suspensions were added into HeLa cells with the concentration varying in the range of 0.5−100 μg/mL. Then they were cultured for 24, 48, and 72 h. HeLa cells in the culture medium devoid of these materials were used as the control. After their incubation, 15 μL of MTT stock solution (5 mg/mL) was added to each well, then the plates were further incubated at 37 °C for 4 h in 5% CO_2 humidified incubator. This resulted in the formation of purple-colored formazan crystals in the living cells, then the supernatant was removed gently, and the purple products were lysed with 150 μL dimethyl sulfoxide (DMSO). The plate was homogeneously agitated for 10 min on a shaker. Then, the absorbance was measured at a wavelength of 490 nm using a microplate reader. Cell viability was expressed as a percent of the control group.

2.5 Observation of nuclear morphology

Nuclear morphology of HeLa cells was examined via fluorescence microscope (OLYMPUS ZX71 TH4-200, Japan) after staining the cells with fluorescein dye: 33342 kit (Beyotime Jiangsu, China). HeLa cells were incubated in 24 well plates using media containing different concentrations of SWNT-COOH/CTAB at 37 °C for 48 h. After thoroughly washing them twice with PBS, the buffer supplied in the kit was added to each well. The cells were stained with Hochest33342 (5 μg/uL) at 4 °C for 20 min and kept in dark. Then, the cells were observed by a fluorescence microscope (OLYMPUS ZX71 TH4−200, Japan). Apoptotic cells were identified through the nuclear morphology changes.

2.6 FITC-annexin V and PI double staining

HeLa cells were used to examine the apoptosis/necrosis induced by SWNT-COOH/CTAB solution after being exposed for 48 h. HeLa cells were seeded at 1×10^5 per well in six well plates, after being incubated for 48 h with the varying concentrations of SWNT-COOH/CTAB. Cell apoptosis was detected by annexin V-FITC apoptosis detection kit (Beyotime, China) according to the manufacturer's instructions. Briefly, cells were gently trypsinized with EDTA-free trypsin. Digestion was terminated by adding DMEM media containing 2% bovine serum albumin (BSA), and the resultant mixture was centrifuged at 2000 r/min for 5 min. Then, it was washed twice with PBS and re-suspended in binding buffer. Finally, it was incubated with annexin V-FITC at room temperature in dark for 10 min. Then, it was immediately analyzed using flow cytometer (Fc 500, Bakerman, America).

2.7 Statistical analysis

Each experiment was repeated triple. Statistical analysis was performed using software SPSS17.0. Data were expressed as mean ± standard error. Statistical significance was determined using one-way analysis of variance and Dunnett's multiple comparison test. The probability values of $P<0.05$ were considered to be statistically significant.

3 Results and discussion

3.1 Characterization comparison of SWNTs and SWNT-COOH

X-ray diffraction(XRD) patterns of the SWNTs and SWNT-COOH sample suggest that SWNTs contained more kinds of impurities compared with SWNT-COOH (Fig. 1). As shown in Fig. 2, the TGA curve suggests that 5% residue of SWNTs was obtained after heating the samples to 600 °C. At such high temperature, we can see that SWNT-COOH is almost burnt away completely. In other words, the 5% residue might be attributed to the catalysts residues mixed in the SWNTs sample. In order to verify the composition of these impurities after heating to 600 °C, we collected the residues after applying TGA and performed quantitative analysis of the sample using energy disperse spectroscopy (EDS) (Fig. 3). The results indicate the presence of various impurity elements in the carbon nanotubes.

In order to investigate the cytotoxity of carbon nanotubes, their manufacturing techniques should be taken into consideration. Several different methods of synthesis, such as laser vaporization, electric arc discharge, and chemical vapor deposition (CVD), were employed to produce carbon nanotubes [19]. However,

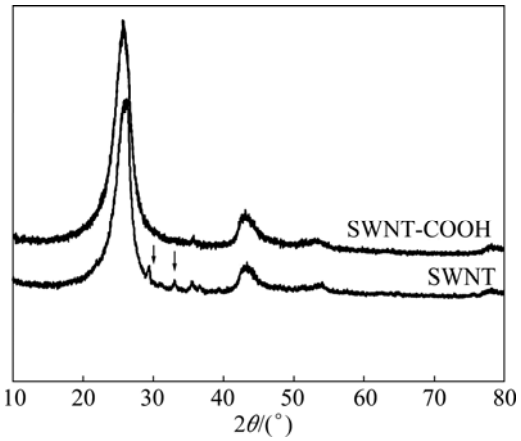

Fig. 1 XRD patterns of SWNTs and SWNT-COOH (Arrows show that SWNTs contain more kinds of impurities.)

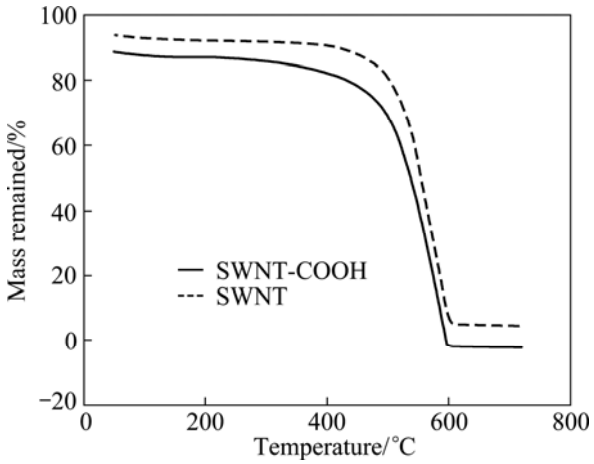

Fig. 2 TGA curves of SWNTs and SWNT-COOH

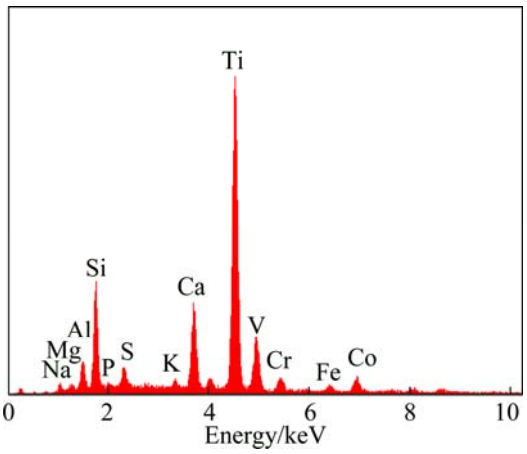

Fig. 3 EDS spectrum of SWNT

all these methods of production still used various catalysts which were hard to remove completely. The catalyst impurities contributed to the cytotoxicity of CNT [20]. Purified CNTs reduced the cytotoxicity and catalysts impurities contained in CNT [21]. Studies [12,22] have found that acid treatment can lead to the

generation of carboxyl (—COOH) group. Carboxylic acid functionalized carbon nanotubes are widely used. By this acid functionalization method, the dispersion, solubility and hydrophilicity of SWNTs in suspension increased, and their catalyst impurities decreased greatly [22]. Our characteristic results indicated that most catalyst impurities were removed by carboxyl acid treatment. This agrees with the findings of the study conducted by ZHU et al [11].

3.2 Cell viability analysis

The MTT assay results of HeLa cell incubation with SWNTs and SWNT-COOH at the concentrations ranging from 0.5 to 100 μg/mL for 48 h are shown in Fig. 4. It can be seen that SWNTs are more toxic than SWNT-COOH in a dose-dependent manner. There is a significant decrease in cell viability at the concentration above 25 μg/mL with SWNTs. In contrast, no significant decrease in cell viability was observed with SWNT-COOH at the concentration of 0.5−25 μg/mL.

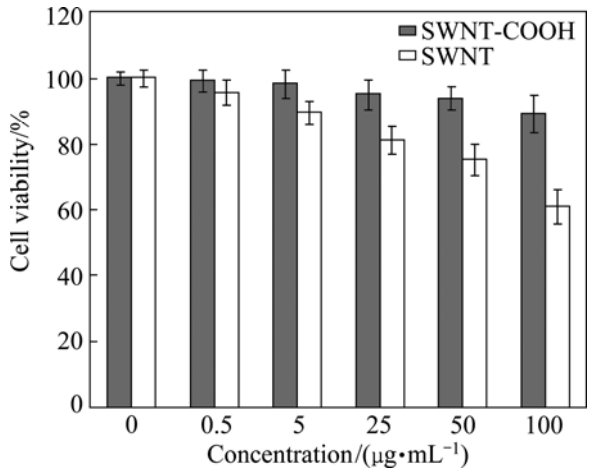

Fig. 4 HeLa cell viability assessment with MTT assay after 48 h exposure to SWNTs and SWNT-COOH

SWNT contains more catalysts than SWNT-COOH and the results show that SWNTs are more toxic than SWNT-COOH. So we suggest that the cytotoxicity of the catalysts contributes to the cytotoxicity of SWNT, which is in agreement with the result of KAISER et al [21].

Figure 5 illustrates that CTAB and SWNT-COOH/CTAB decrease the cell viability in concentration-dependent manner. The cytotoxicity of SWNT-COOH/CTAB is more related to the toxicity of the surfactant cetyltrimethyl ammonium bromide. At low concentration levels of 0.5−25 μg/mL, the toxic effect of CTAB and SWNT-COOH/CTAB is acceptable. The cytotoxicity of the SWNT-COOH/CTAB is more related to the toxicity of the surfactant CTAB.

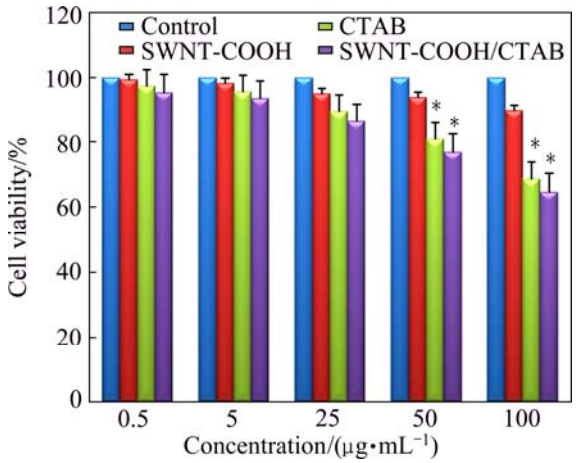

Fig. 5 Comparison of cell viabilities (Three kinds of materials were exposed to HeLa cells at concentration of 0.5−100 μg/mL for 48 h. Statistical analysis was performed with one-way ANOVA analysis and Dunnett's comparison tests; vs control group, *P<0.05)

In order to further investigate whether SWNT-COOH/CTAB has both time and concentration dependence, HeLa cell viabilities of SWNT-COOH/CTAB in concentration-dependent doses of 0.5−100 μg/mL and time-dependent course of 24, 48, and 72 h are shown in Fig. 6. It can be seen that the concentration was varied significantly between 50−100 μg/mL for 48 h and 72 h. The cell viability of SWNT-COOH/CTAB decreased with the increase of the exposure time, but the decrease came to ease after 48 h.

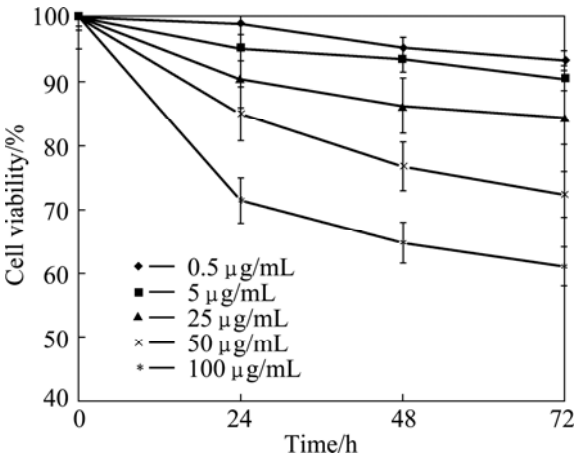

Fig. 6 HeLa cell viabilities after being exposed to SWNT-COOH/CTAB in concentration-dependent doses of 0.5−100 μg/mL through time-dependent course of 24, 48, and 72 h

International Standardization Technical Committee established the biological evaluation of ISO10993 international standards [23]. The evaluation imposed strict standards for the safety evaluation of biological materials. Cytotoxicity assessments of carbon nanotubes were complicated because various factors, such as purity,

surface chemistry, surface area, aggregation, catalyst and soot-like contamination have different synergistic impacts on the cytotoxicity effects [24,25]. So, the assessment of cytotoxicity of nanotubes is a complicated process. KAISER et al [21] found that purified SWNTs were less toxic than SWNT pristine materials. PULSKAMP et al [22] concluded that acid-treated SWNT containing less metal catalysts could reduce intracellular reactive oxygen species. Based on the characterization analysis of SWNT and SWNT-COOH, the results suggest that the toxicity of SWNTs is highly dependent on the metal catalysts impurities, which is in good agreement with the results of Refs. [21,22]. The acid functionalization improves the solubility and dispersion of carbon nanotubes in suspension and decreases the cytotoxicity [26]. But ZHU et al [11] found that multi-walled carbon nanotubes were more toxic than the MWNTs. It is presumed that the different structures between multi-walled carbon nanotubes and single-walled carbon nanotubes might contribute to the different cytotoxicity, or maybe different cell line shows difference.

CTAB is a widely used surfactant in industrial field. Our previous work suggested that CTAB could well conjugate with nuclear acid as a promising surfactant and be modified with SWNT for gene transfection. Our cytotoxicity results showed that the cytotoxicity of SWNT-COOH/CTAB was acceptable at low concentrations of 0.5−50 μg/mL according to the ISO10993 international standards [23]. This was similar to the results reported by ZHANG et al [17]. They found that CTAB-PBCA NPs (cetyltrimethyl ammonium bromide-polybutylcyanoacrylate nanoparticles) were harmless to HepG2 cells when the concentration of NP solution was less than100 ng/μL.

3.3 Cell apoptosis analysis

HeLa cells stained with Hochest33342 fluorochrome, after a 48 h exposure to SWNT-COOH/CTAB at different concentrations (25−100 μg/mL) are shown in Fig. 7. The microscopic picture shows that the control cells (untreated group) present homogeneous blue nuclei chromatin (Fig. 7(a)). HeLa cells grow and spread well in the control group. In contrast, cells exposed to SWNT-COOH/CTAB show bright blue nuclei, typical apoptosis characteristics, such as cell shrinkage, nuclear condensation (Figs. 7(c) and (d)). These nuclear morphological features are remarkable when these cells are exposed to SWNT-COOH/CTAB at the concentration of 100 μg/mL. In fact, some cells become round in shape and the number of cells decreases, whereas others undergo autolysis (Fig. 7(d)).

As shown in Fig. 8, little apoptotic cells were detected in the control group, and a concentration-dependent increase in apoptosis was found with SWNT-COOH/CTAB, especially up to the concentration of 100 μg/mL. But, at low concentrations (0.5−25 μg/mL), the apoptosis effect of CTAB is acceptable in accordance with the findings of the MTT assay. Annexin V- FITC is positive while the remaining propidium iodium (PI) is negative. This is the characteristic of an early stage of apoptosis. phosphatidylserine (PS) exposure on the outer surface of the plasma membrane does not cause cell membrane damage. In contrast, PI is used to distinguish necrotic cells from apoptotic and living cells. The rates

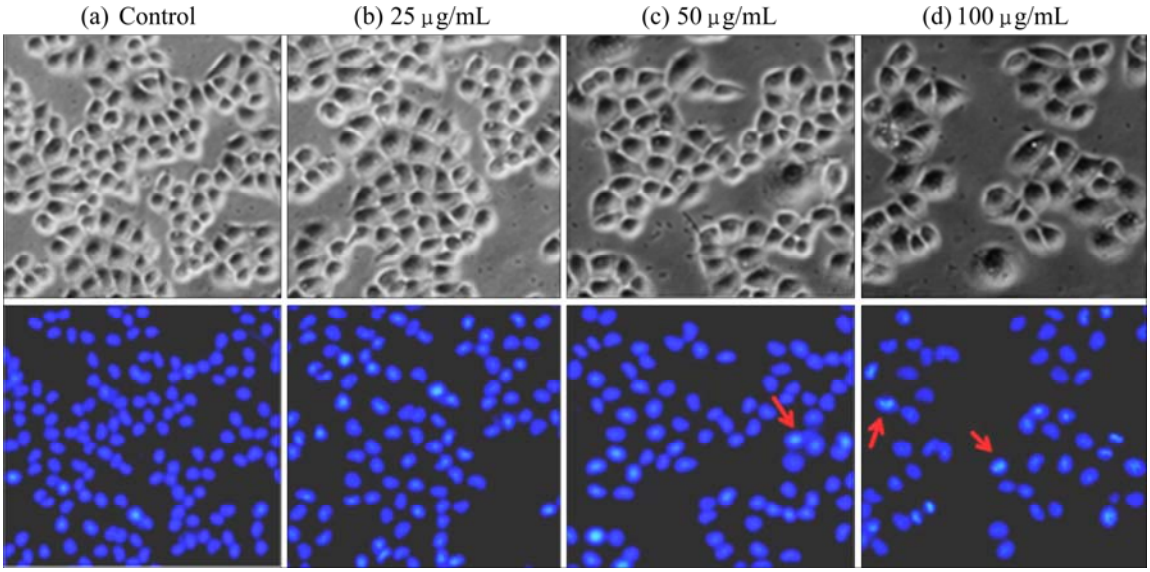

Fig. 7 Morphological changes of HeLa cells detected in optic micrographs (40*) and fluorescent micrographs (100*): (a) Control; (b) SWNT-COOH/CTAB: 25 μg/mL; (c) SWNT-COOH/CTAB: 50 μg/mL; (d) SWNT-COOH/CTAB: 100 μg/mL (Bright blue nuclei and condensed chromatin, deformed nuclear size marked by arrows)

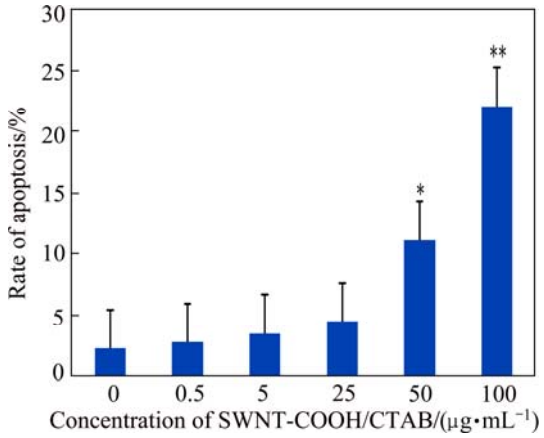

Fig. 8 Percentage of apoptotic cells exposed to SWNT-COOH/CTAB for 48 h with concentration of 0−100 μg/mL by flow cytometry; vs control group, **P*<0.05, ***P*<0.01

of living, apoptotic and necrotic cells were detected by annexinV/PI double staining and analyzed by flow cytometry. At low concentrations (0.5−25 μg/mL), HeLa cells suffer negligible toxic and apoptotic effects when exposed to SWNT-COOH/CTAB. On the other hand, at higher concentrations (50−100 μg/mL), the apoptotic effects become apparent.

SWNTs can induce cell apoptosis, which might be due to the oxidative stress [8,27]. It can be found that the SWNT shows cytotoxicity effect by MTT assay for it contains more catalysts. The apoptosis analysis of SWNT-COOH/CTAB shows concentration-dependent cytotoxicity at higher concentrations. When SWNTs are added into the culture media containing the fetal bovine serum and other nutrients, the culture media interact or absorb the SWNTs [28], or the SWNT alters the composition of the culture medium [29]. So, with higher concentration, the media constituent cannot totally attach to SWNTs, and the cytotoxicity of SWNT-COOH/CTAB is more related to the toxicity of the surfactant molecules cetyltrimethyl ammonium bromide(CTAB). Therefore, maybe at higher concentrations, the toxicity of the residue SWNT- COOH/CTAB appears obviously.

4 Conclusions

1) SWNTs contain more kinds of impurities compared with acid-purified SWNT (SWNT-COOH). SWNTs are more toxic than SWNT-COOH. Carboxylic acid functionalized carbon nanotubes can greatly decrease cytotoxicity.

2) CTAB and SWNT-COOH/CTAB decrease the cell viability in a dose and time-dependent manner. The cytotoxicity of SWNT-COOH/CTAB is more related to the toxicity of the surfactant cetyltrimethyl ammonium bromide(CTAB) and the cytotoxicity of CTAB and SWNT-COOH/CTAB is acceptable at low concentration (0.5−25 μg/mL).

3) SWNT-COOH/CTAB behaves dose-dependent cell apoptotic effects to HeLa cells. At low concentration (0.5−25 μg/mL), HeLa cells suffer negligible toxic and apoptotic effects when exposed to SWNT-COOH/CTAB.

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十六烷基三甲基溴化铵修饰的羧基化单壁碳纳米管的细胞毒性

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摘 要：采用十六烷基三甲基溴化铵(CTAB)作为表面活性剂修饰羧基化的单壁碳纳米管(SWNT-COOH/CTAB)，并对原始单壁碳纳米管(SWNTs)与羧基化修饰的单壁碳纳米管(SWNT-COOH)进行材料学特征比较。通过细胞活力和细胞凋亡实验对 SWNTs、SWNT-COOH 和 SWNT-COOH/CTAB 的细胞毒性进行比较。结果表明，羧基化修饰的单壁碳纳米管比原始单壁碳纳米管的毒性小，单壁碳纳米管经羧基化后其毒性降低；浓度及时间曲线显示 SWNT-COOH/CTAB 的毒性与表面活性剂 CTAB 相关，CTAB 和 SWNT-COOH/CTAB 的细胞毒性在低浓度范围内(0.5~25 μg/mL)是可接受的。十六烷基三甲基溴化铵修饰的羧基化单壁碳纳米管在低浓度范围(0.5~25 μg/mL)内可以较安全地用于生物医学领域。

关键词：碳纳米管；十六烷基三甲基溴化铵；毒性；酸化；凋亡

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