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THE ANTIBACTERIAL ACTIVITY OF COVALENT-FUNCTIONALIZED CARBON NANOTUBES AGAINST *ESCHERICHIA COLI*

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Single-walled carbon nanootubes (SWCNTs) were conjugated with hydroxyl, carboxyl and amino groups. Fourier transform infrared measurements validated the presence of a covalent linkage between SWCNTs and chemical groups. The antibacterial activity of functionalized SWCNTs was evaluated by standard plate count method, and the transformation in micromorphology of the strains, *Escherichia coli*, induced by covalent-functionalized SWCNTs was achieved using scanning electron microscope. The results indicated that pristine SWCNTs with high metal impurity content showed more active in antimicrobial activity than SWCNTs purified by diluted nitric acid. After the SWCNTs were functionalized by hydroxyl groups, the antibacterial activity was increased compared to pristine and purified carbon nanotubes. The sequence of antibacterial activity was SWCNTs-OH > raw SWCNTs > purified SWCNTs > SWCNTs-COOH > SWCNTs-NH_2 , which was in accordance with the order of absolute value of zeta potential. It is the first time to report that antibacterial activity of SWCNTs is zeta potential dependent. The antimicrobial activity was attributed to both functional groups and metal impurity. The transformation of bacterial cells was studied using electron scanning microscopes. After interaction with SWCNTs, the cellular morphology of *E. coli* was flat, and the strain died.

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Keywords: SWCNTs, FTIR, TGA , Zeta potential

1. Introduction

In 1991, Iijima [1] accidentally discovered carbon nanotubes for the first time when he observed the structure of fullerene under polymer transmission electron microscopy with arc discharge method. Carbon nanotubes consist exclusively of carbon atoms arranged in a series of condensed benzene rings rolled-up into a tubular structure. As a crystalline form of carbon allotropes, carbon nanotubes showed the nature of one dimension compared with fullerene of zero dimension, graphite of two-dimension and diamond of three-dimension. Due to their remarkable and unique mechanical, electrical, optical and thermal properties, carbon nanotubes, especially single-walled carbon nanotubes (SWCNTs) have captured a great deal of attention in in both academia and industry research as superconductors, optical devices, sensors, energy storage devices, fuel cells and catalyst manufacturing [2]. With the development of nanotechnology, carbon nanotubes have been exploiting numerous biomedical applications such as drug carrier [3], biosensor [4] and tumor hyperthermia [5]. Despite the widely application of carbon nanotubes, a number of studies have been reported on the potential toxic effects of the nanomaterials to lungs [6] and human cells [7]. On the other hand, the toxic carbon nanotubes were used to kill undesirable microorganisms. In the last few years, the discovery of antibacterial activity of carbon nanotubes triggered a new research in biomedical field [8]. Research indicates [9] that due to their unique structure and size, carbon nanotubes showed good bactericidal and bacteriostatic activity when

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bacteria get thoroughly exposed to them, and the antibacterial activity were closely related with its size and specific surface area[10, 11], for this reason, single-walled carbon nanotubes (SWCNTs) displayed stronger antibacterial activity than multi-walled carbon nanotubes (MWCNTs) [9-11].

SWCNT is one of the most concerned for its unique physicochemical properties. However, SWCNTs tend to aggregate and the insolubility in solvents limited their extensive application. In order to obtain a fine dispersion, covalent or non-covalent methods were developed to break the cohesion of aggregated carbon nanotubes. Various functional groups can be introduced onto the surface of carbon nanotubes. Meawhile, a functionalized carbon nanotube might be different from those of the original nanotube in the properties of their surface depending on functional groups. Similarly, the adverse effects of carbon nanotubes on biological systems do not only depend on the intrinsic toxicity of carbon nanotubes and the different functional groups [12].

In this study, the effects of different functional groups (-OH, -COOH and $–NH_2$) combined on the surface of SWCNTs were investigated on antimicrobial activity against Gram-negative bacterium *Escherichia coli.* It was found that SWCNTs functionalized by acovalent method with different groups produced different cytotoxic effects on bacterial cells. However, the antibacterial activity of SWCNTs does not only depend on sidewall functionalization but the impurities contained after synthesis in pristine SWCNTs.

2. Materials and methods

2.1 Chemicals and materials

All SWCNTs were purchased by Chengdu Organic Chemicals Company Ltd. of Chinese Academy of Sciences, which were produced by chemical vapor deposition (CVD). Reagents in the experiments were all in analytical grades.

2.2 Preparation of SWCNTs

Purified SWCNTs (p-S) were obtained as follows. Raw SWCNTs (r-S) (40 mg) were added into 80 ml nitric acid (1 mol/L) and dispersed in ultrasonic for 4 h in ice bath. The resulted materials were washed by distilled water to neutral and then dried.

In order to produce SWCNTs-OH (S-OH), ten milligrams of p-S were dispersed in 4 ml of $FeCl_2$ solution (0.5 M, pH 3.0) and sonicated for 15 min, and then 2 ml of 30% H_2O_2 was added drop by drop [13]. The resulting suspension was sonicated for 2 h and filtered. The filtrate waswashed withultrapure water to neutral and dried..

The carboxyl groups were derived from the surface of SWCNTs by acid treatment. Twenty milligrams of p-S was added into 40 ml nitric acid (7 mol/l) in reflux condenser under 110 ℃ for 12 h [14, 15]. The oxidized SWCNTs were washed with ultrapure water to neutral and dried to gain SWCNTs-COOH (S-COOH).

The $-NH_2$ groups were conjugated with SWCNTs by acylation of the carboxyl moieties on S-COOH with 1-(3-(Dimethylamino)propyl)-3-ethyl carbodiimide hydrochloride (EDC.HCl) and subsequent reaction with ethidene diamine in dark.

2.3 Characterization of SWCNTs

Characterization of prepared SWCNTs included Fourier Transform Infrared Spectrometer (FTIR) (Nicolet iS10, Thermo Fisher Company) to validate the conjugated groups, laser nanometer (NANO-ZS90, Malvem Instrument Company) and thermogravimetric analysis (TGA-50, SHIMADZU).

Thermogravimetric analysis (TGA) was introduced to determine impurities of SWCNTs. A SWCNT sample was placed in a platinum pan in the 40 sccm air flow and heated up to 800 °C at a 10 °C/min ramp. To correct the TGA profile, the process was repeated after the system was cooled to

room temperature to collect data serving as the baseline.

2.4 Cultivation of strains

E. coli strains were provided by the Institute of Food and Drug Control of Henan Province. The bacteria were cultured on beef extract peptone agar medium at 37 ℃ for 16 to 18 h and all harvested by normal saline (0.9%). Bacterial suspensions were diluted in saline to obtain cell samples with concentration ~10^7-10^8 cfu/ml by 0.5 McFarland standards.

2.5 Evaluation of antibacterial activity of SWCNTs

Aliquots of 1 ml of prepared bacterial suspensions were introduced into 3 ml bottles. One milliliter of desired SWCNT solutions at the concentration of 0.5 mg/ml was added. The equal mixture of saline and bacteria was as control.

The mixtures were cultivated at 37 ℃ and shaked at 175 rpm for designed treatment times .

The reduction in viable cell number after interaction was determined by the traditional plate count method. Each sample was taken 0.1ml and diluted serially (1:10) with normal saline and spread onto beef extract peptone agar plate. Colonies were counted after 24 h incubation of the plates at 37 ℃. It was executed in triplicate.

The formula to calculate the bacteriostatic rate is as following:

Bacteriostatic rate （100%）= (1- colonies of test groups/ colonies of control group)×100.

2. 6 Scanning electron microscopy imaging

Scanning electron microscopy (SEM) (Tescan, VEGA Ⅱ LMU) was introduced to observe the morphology of bacteria. Samples were fixed with 2.5% glutaraldehyde and 1% osmium tetroxide and prepared by putting a 1 μl droplet on a silica chip and air drying. The cells were coated with gold (30 s, 30 mA) for SEM imaging.

2.7 Statistical analysis

Analysis of variance (ANOVA) was used to analyze data by data processing system.

3. Results

3.1 Determination of grafting groups based on FTIR spectrogram

As shown in the spectra of r-S (Fig. 1a), two apparent peaks were at 3436 and 1574 cm^{-1}. The first broad peak at 3436 cm^{-1} displayed that hydroxy (–OH) existed on SWCNTs. It meant that hydroxyl was grafted in the process of SWCNT production. The peak of 1574 cm^{-1} was the characteristic absorption peak of carbon nanotubes, which was the proof of the graphite structure existence in carbon nanotubes [14].

The spectra of purified SWCNTs (p-S) were shown in Fig. 1b. Three peaks (1574, 1720 and 3436 cm^{-1}) were displayed. The absorption peak of 1720 cm^{-1} was characteristic vibration frequency of carbonyl group (–C=O), and the broad peak of 3436 cm-1 was hydroxyl (–OH). Thus, carboxyl groups were bound on the surface of SWCNTs. It was revealed that diluted nitric acid was helpful to functionalize carbon nanotubes by covalent binding of carboxyl groups.

Fig. 1c displayed the FTIR spectra of S-OH. The characteristic peak of hydroxyl (–OH) spanned from 3200 to 3500 cm^{-1}, which meant hydroxyl groups were successfully bound.

In the spectra of S-COOH (Fig. 1d), the peak at 1717 cm^{-1} and the broad one at 3436 cm^{-1} were indicated that the samples was functionalized by carboxyl groups.

Three absorption peaks of S-NH_2 were shown in Fig. 1e. The peak at 1630 cm^{-1} was due to bending vibration of amino-groups (–NH_2). The double peaks caused by stretching vibration of amino-group should appear between the wavenumber of 3200 and 3400 cm^{-1}. But they were not found in these spectra. It maybe resulted from the same absorption area with hydroxyl.

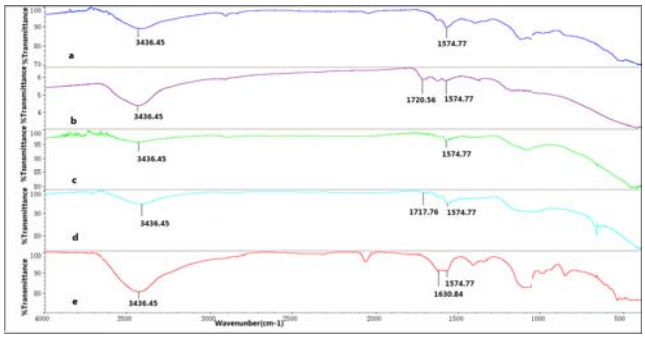

Fig.1 FTIR spectra of SWCNTs modified by chemical groups Note: a: raw-SWCNTs (r-S), b: purified SWCNTs (p-S), c: SWCNTs-OH (S-OH), d: SWCNTs-COOH (S-COOH), e: SWCNTs-NH_2 (S-NH_2)

3.2 The changes of SWCNT Zeta potential after functionalization

The results showed that the surface charge of SWCNTs was negative, and the zeta potential was changed after functionalization (Fig. 2). The potential of pristine SWCNTs was near to -5 mV. The electrostatic repulsive forces were decreased by purification, functionalization of carboxyl and amino groups, which were characterized by the decrease of zeta absolute values. But for hydroxyl attachment, it was reverse. The zeta potential was almost reached to -18 mV. The sequence of absolute potential was S-OH > r-S > p-S > S-COOH > S-NH_2.

After standing for 24 h, aggregation state was observed in all solutions (Fig. 3). And particle diameters were far higher than 100 nm (data not shown), it also illustrated that majority of SWCNTs existed in bundles.

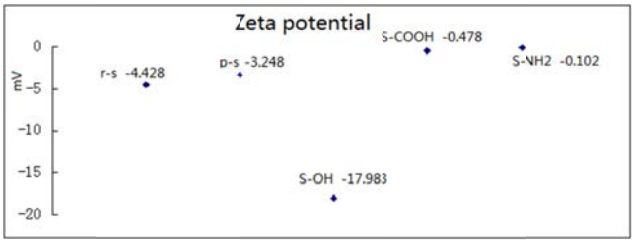

Fig. 2 Zeta potential of SWCNTs (pH 7.0)

Fig. 3 SWCNTs dispersed in ultrapure water
Note: a: r-S, b: p-S, c: S-OH, d: S-COOH, e: S-NH_2; SWCNT concentration: 0.5mg/ml, standing for 24 h after dispersed by cell disruptor in ice bath.

3.3 The thermal stability of SWCNTs

The thermal properties of SWCNTs were detected by TGA. The results were shown in Fig. 4. The data revealed that the oxidation temperature of r-S was about 620 ℃, and when the samples was heated to 800 ℃, 10.58% of original mass were remained. After purification, the temperature increased (661 ℃). Besides, the residues were less (5.28%) than that of r-S. That suggested thermal stability of purified SWCNTs was improved. The excellent thermal stability indicated that the purified SWCNTs were neither damaged nor derivatized by the purification process.

For covalent modification SWCNTs, a small amount of weight loss occurred below 550 ℃ resulted from the losing of grafting groups bonded to the surface of SWCNTs. The onset temperature all decreased compared to purified SWCNTs, which may be attributed to the breakdown of the surface too. The mass of residues was dramatically lower than that of p-S. It was inferred that metal catalysts were removed partially during covalent process.

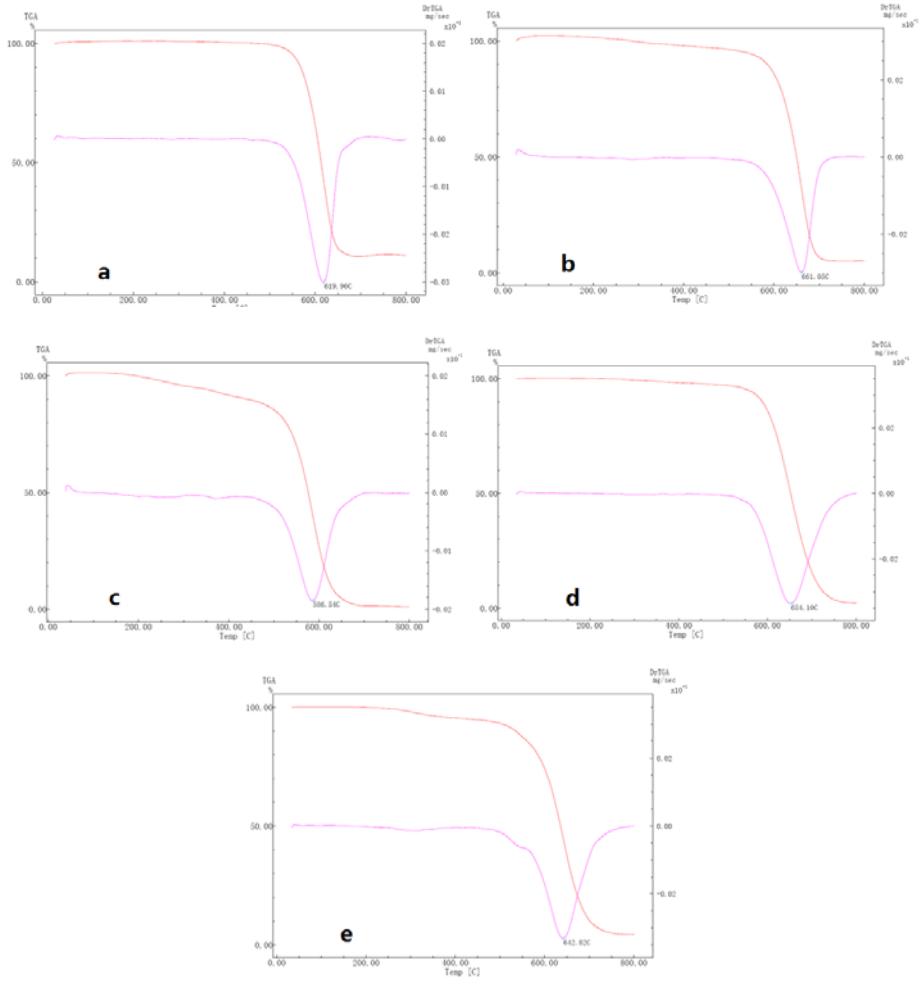

Fig. 4 Thermogravimetric curve of SWCNTs
Note: Thermogravimetric analysis of 1-2 mg samples ramped from 25 to 800 ℃ at 10 ℃ per min in a platinum sample pan under 40 sccm flowing air. a: raw-SWCNTs (r-S), b: purified SWCNTs (p-S), c: SWCNTs-OH (S-OH), d: SWCNTs-COOH (S-COOH), e: SWCNTs-NH_2 (S-NH_2)

3.4 Antibacterial activity of SWCNTs against E. coli

The results were shown in Fig. 5. *E. coli* served as a model of microbes for assessing the bacterial toxicity of SWCNTs. The antibacterial activity of SWCNTs with different surface groups

was estimated by counting the numbers of colonies growing on the plates.

It was found that the growth of *E. coli* was inhibited after exposure to SWCNTs, which were included r-S, p-S, S-OH, S-COOH and S-NH_2. As results indicated, more than 99% of bacteria *E. coli* were killed after treatment with both r-S and p-S for 8 h. At the terminal of the experiment, the bactericidal rate caused by r-S and p-S was 99.92% and 99.6%, respectively. Although they were not much different in bactericidal rates in the end of experiment, the time to kill the microorganism was not consistent. The toxicity of r-S was shown faster than p-S. The difference was mainly occurred in the initial of exposure. After 1 h of interaction with r-S, 66.92% of strains *E. coli* were dead; meanwhile, only 12.30% of the strain was inactivated induced by p-S. Four hours later, the bactericidal rates were similar (96.76% for r-S and 95.95% for p-S).

As for functionalized SWCNTs (S-OH, S-COOH and S-NH_2), different antibacterial activities against *E. coli* were revealed. In the first hour, toxicity of SWCNTs was performed in different speed. S-OH displayed antimicrobial activity at the earliest time, and followed by r-S, S-COOH, p-S and S-NH_2 in sequence. Moreover, S-OH showed the strongest bactericidal activity. It was significantly different compared to the other two ($P < 0.05$). After exposure to S-OH for one hour, more than 80% of bacteria *E. coli* were killed. The mortality rate was only 30.54% and 7.24% induced by S-COOH and S-NH_2, separately. Eight hours later, only less than 1% of bacteria were vital (fig.5b) at the end of the experiment. At the same time, 71.71% of *E. coli* was inactive caused by S-COOH, and 74.90% by S-NH_2.

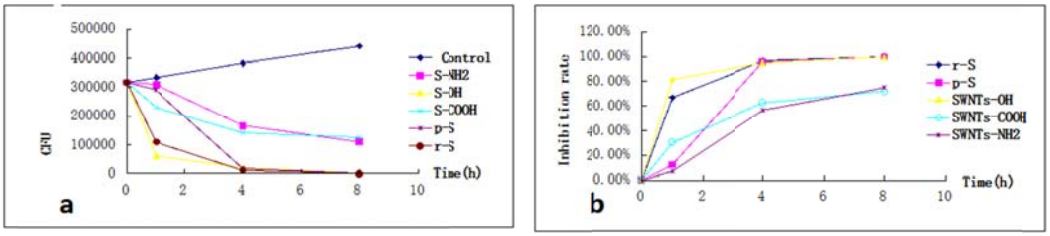

Fig. 5 Antibacterial activity of SWCNTs
Note: (a) Growth curves of E. coli (CFU) in 0.9% NaCl at 37 ℃ after the cells were treated with SWCNTs (0.25 mg/ml) (b) Inhibition rates of E. coli by SWCNTs.

3.5 Scanning electron microscopy (SEM) images of E. Coli

Fig. 6 showed SEM images of *E. coli* in vital shape (Fig. 6a) and the bacterial cells interacting with SWCNTs (Fig. 6b). In living state, the cell of *E. coli* was rod-shaped and the length was about 2-3μm (Fig. 6a). After interaction with SWCNTs, in comparison of untreated bacterial cells, the cells of *E. coli* was flat and lost its cellular integrity (Fig. 6b) induced by SWCNTs. The morphological transformation was most likely due to damage to the membrane structures of bacterial cells.

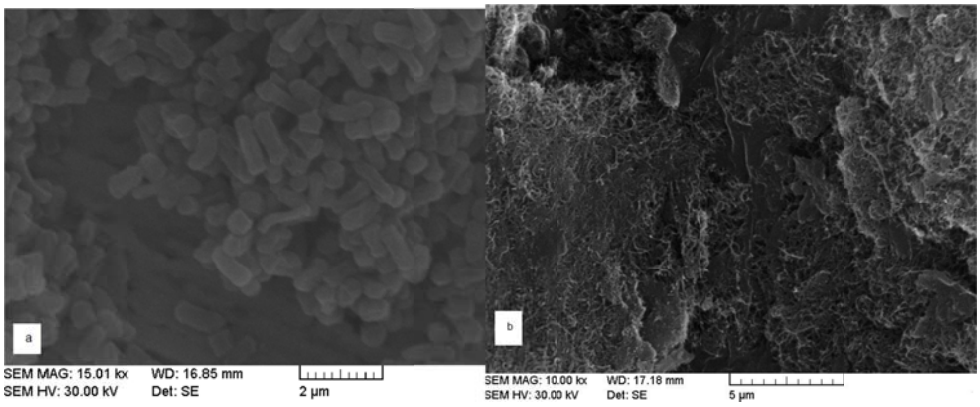

Fig.6 Scanning electron microscopy images of E. coli cells (a) without SWCNTs (b) with SWCNTs

4. Discussion

In this experiment, SWCNTs used were produced by CVD. In the process of production, hydroxy groups (-OH) were conjugated on the surface of SWCNTs, and some impurities were mixed with the SWCNTs, which mainly included metal catalysts. A quantitative measure of residual metal catalyst in nanotube samples was provided by TGA. In the results, initial mass loss was attributed to the oxidation of amorphous carbon impurities and groups combined on the surface of SWCNTs when the temperature was less than 500 ℃ rather than nanotubes themselves. SWCNT combustion occurred at a higher temperature from 500 ℃ to 700 ℃. Upon heating to 800 ℃ in air, metals were left [16]. Consistent with others [17], defects and derivatization moiety in nanotube walls can lower the thermal stability. After covalent functionalization, the oxidation temperature of SWCNTs decreased compared to p-S. The purification (acid treatment) not only removed some of metals but also produced carboxyl functional groups. We also reported oxidants (HNO_3, $KMnO_4$ and H_2O_2) could combine carboxyl groups on the surface of SWCNTs [14].

Metal may cause the faster toxicity of r-S than p-S to *E. coli*. The pristine nanotubes (r-S) were mixed more metals than the sample p-S. Metal could effectively induce the production of free radicals. The oxygen radicals excessively gathered and led to cell membrane function obstacle, protein degradation, DNA damage and abnormal signal transduction [18]. Similar results were reported in Diamond and Related Materials [19]. In the first hour of incubation, it was metal that worked as bactericidal agents and attributed to faster toxicity. The toxicity of pristine SWCNTs was also reported by Lacerda's group. Batches of pristine SWCNTs (non-purified and/or non-functionalised) readily after synthesis contain impurities such as amorphous carbon and metallic nanoparticles (catalysts: Co, Fe, Ni and Mo) can be the source of severe toxic effects[12].

Covalent-functionalized SWCNTs changed the structure and electronic properties of SWCNTs to a certain extent [20]. The functionalization procedures alter the physicochemical properties of SWCNTs significantly enough to change SWCNT cytotoxicity in *E. coli*. Our results were similar with previous reports [21]. SWCNTs modified with -OH, -COOH and -NH_2 groups showed various antimicrobial activities. Compared to r-S and p-S, bactericidal activity of S-OH enhanced, and the other two displayed lower activity. However, multi-wall carbon nanotubes with –OH and –COOH groups did not exhibit antibacterial activity [21]. Different groups combined on the surface of SWCNTs may be the key point attributed to the different activity against *E. coli* among all three types of SWCNTs. The carboxyl and amino groups were grafted on the surface defects of SWCNTs, which were organic fuctionalization and produced more defects on the surface walls of SWCNTs [22]. At the same time, hydroxyl functionalization was mild and little damage to the surface of SWCNTs, which was non-organic functionalization [23]. The highest inactive rate of bacterial cells may be resulted from the little surface damage of SWCNTs. Arias [21] explained the difference of antibacterial activity that S-OH and S-COOH were derived directly from the SWCNT surface and could contact directly with SWCNTs, while S-NH_2 functionalized with the long chain of carbon may not be in close direct contact with the cell walls. In the relationship of SWCNTs and groups, higher zeta potentials occur for smaller molecular size. Thus, SWCNTs with small tail groups may show strong antibacterial activities.

For electrostatic reasons, colloidal particles with $|\zeta| > 15$ mV are expected to be stable. Thus, to achieve stable nanotube dispersions, the zeta-potential should be maximized [24]. In this article, only the potential of S-OH was more than 15 mV. Because of the large mutual attractions, S-OH also tends to aggregate and deposit. Based on the results of this experiment, SWCNTs with higher absolute values of zeta potential were stronger bactericidal activity. In another word, the loss of viability of *E. coli* increased in the interaction solutions as the quality of nanotube dispersion was improved. This implied that decrease of bacterial viability is critically linked with nanotube dispersion quality. Previous studied reported that the likely mechanism of bactericidal activity of SWCNTs is direct contact with bacteria [25]. SWCNTs in high-quality disperse have more chances to interact with bacterial cells directly than aggregated ones. This result is in accordance with previously published data [26].

From a charge perspective, although the cell wall of *E. coli* is negatively charged,

SWCNTs with high negative potential induced a significant loss of viability on *E. coli*. The similar results were published in Langmuir about antimicrobial activity of SWCNTs against *Salmonella typhimurium*, a rod shaped Gram-negative bacterial pathogen [21]. It was different from fullerene [27] and metal oxide nanoparticles (TiO_2) [28]. Cationic fullerene and TiO2 nanoparticle generally exhibited stronger antimicrobial activity than anionic ones. It can be inferred that the surface charges of SWCNTs and microorganisms may not a critical point during interactions.

The SEM photos proved that after interaction with SWCNTs, *E. coli* lost the cellular integrity and vitality, and it is consistent with the results of Kang [9]. Bai [29] found SWCNTs can not only capture cells, but also cause cell death due to the direct physical puncture, resulting in damages to the outer membrane of cells. Brady-Estévez et al. [30] also proved that SWCNTs can lead to bacterial aggregation and deposition, thus cause membrane damage and death. Vecitis et al. [31] also revealed that SWCNTs can cause damage to cell membrane and force bacterial inclusions releasing to kill bacteria. Thus, the morphological changes are most likely due to damage to the membrane structure.

5. Conclusions

The covalent functionalization of SWCNTs with -OH, -COOH and -NH2 and their antibacterial activity against *E. coli* were studied. In the first stage of interaction, metal catalyst mixed in the SWCNTs was the main factor acting on the strain. The sample r-S with more metal impurities showed toxicity faster than p-s with less metal. Covalent modification could change the surface properties of SWCNTs. Covalent functionalization introduced new functional groups to SWCNTs which altered their toxicity and antibacterial properties. After combination of hydroxy group (-OH), SWCNTs displayed highest zeta potential (absolute value) and most bactericidal activity among all groups (-OH, -COOH and -NH2). It was the first time to reveal the antibacterial activity of SWCNTs was zeta potential dependent. The dispersion quality is strongly correlated with zeta potential. Higher absolute zeta potential implies higher quality of SWCNT solutions. SWCNTs with small tail groups should be preferred to achieve high zeta potential. Thus, antibacterial activities of SWCNTs depend on metal impurities, functionalized groups and disperse quality. Its mechanisms are not fully understood yet. Obviously, SEM photos illustrated that death of bacterial resulted from the loss of cellular integrity.

These results indicated that original and modified SWCNTs were all toxic to *E. coli*, and they can be potential as an antibacterial agent and prospectively bring about an end of the bacterial drug-fast.

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