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Microbial toxicity of ethanolamines—Multiwalled carbon nanotubes

Hadi Zare Zardini,[1,2] Morteza Davarpanah,[3] Mehdi Shanbedi,[3] Ahmad Amiri,[4] Morteza Maghrebi,[3] Leila Ebrahimi[5]

[1]Department of Pediatric, Hematology, Oncology and Genetics Research Center, Shahid Sadoughi University of Medical Sciences and Health Services, Yazd, Iran
[2]Young Researchers and Elite Club, Yazd Branch, Islamic Azad University, Yazd, Iran
[3]Department of Chemical Engineering, Faculty of Engineering, Ferdowsi University of Mashhad, Mashhad, Iran
[4]Department of Engineering, Marvdasht Branch, Islamic Azad University, Marvdasht, Iran
[5]Department of Hematology, Iranian Blood Transfusion Organization, Tehran, Iran

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Abstract: In the present study, antimicrobial activities of multiwalled carbon nanotubes (MWCNTs) functionalized with ethanolamine (EA) groups were investigated. Therefore, MWCNT were first functionalized with mono-, di-, and triethanolamine (MEA, DEA, and TEA) under microwave technique. Development of functional groups on the MWCNT surface was confirmed by Fourier transform infrared and thermogravimetric analysis. Morphological variation was investigated by transmission electron microscopy. Then, antimicrobial activities of pristine and functionalized MWCNT (MWCNT-MEA, -DEA, and -TEA) were tested against different bacteria species. The studies have been done on four Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeroginosa*, and *Salmonella typhimurium*) as well as four Gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Bacillus cereus*, and *Streptococcus pneumonia*). The results based on minimal inhibitory concentration and radial diffusion assay were shown that the antimicrobial activity of MWCNT-TEA > MWCNT-DEA > MWCNT-MEA > pristine MWCNT. Based on the results, it seems that EA groups could play an important role in antimicrobial activity of MWCNT.
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Key Words: carbon nanotube, functionalization, antimicrobial, ethanolamines

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INTRODUCTION

Numerous people were annually dying in the worldwide due to infectious diseases.[1] Nowadays, common antimicrobial agents such as kanamycin, and penicillin were applied to solve such infectious diseases. Unfortunately, the antimicrobial activity of such agents neutralizes the desired disinfection function influence of overusage, leading to resistance of bacteria.[2] This truth demonstrates that the public health needs to expand novel antimicrobial agents and drag.[3,4]

Recently, various nanostructures such as metal nanoparticle (NP) and carbon structure have promised to assuage the resistance issue of different strains.[5–7] NP especially metal NPs such as Ag-NPs have higher permeation into surface and ground waters.[8] As a heavy metal, this could aggravate the negative impact of this particle for environmental.

In contrast, carbon nanostructures are less solved, and could decorate with special functional groups in order to enhance novel potential in the disinfection area.[9–11] Among various carbon nanostructures, carbon nanotubes (CNTs) have been recently verified to own positive antimicrobial properties, and their relevant activities were attributed to the physical membrane-damage mechanism.[7,9,12] The former researches demonstrated that CNT showed the effective antimicrobial activity and high potential to defect the diseased pathogens. In agreement with Kang et al.[13] results, with successful accumulation on bacteria's membrane of *Escherichia coli* and making defect on main structure, CNT abolish the bacteria. Another study confirmed that as the length of CNT increase, antimicrobial activity increase too because of the much successful contact with membrane.[14] Needless to say, resistant bacteria could be eliminated by CNT, gradually.[9,15] A variety of factor influenced on the antimicrobial activity of CNT such as length,[14] type of functional groups on the main structure,[9,16] electronics structure,[17] functionalization technique, diameter,[18] type of solvent,[15] and so on.

Meanwhile, CNT illustrated valuable activity against bacteria of digestive system. Decorating different functional groups on the CNT structure proved that the antimicrobial activity increased than that of pristine one.[9,10,19] Adding

Correspondence to: A. Amiri; e-mail: ahm.amiri@gmail.com
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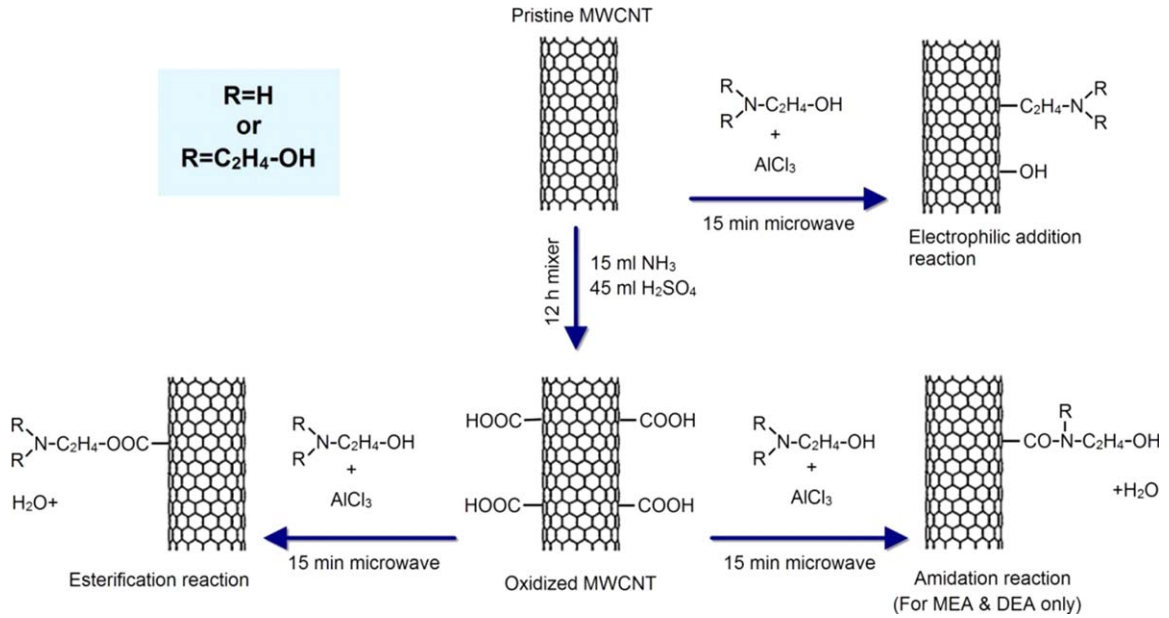

FIGURE 1. Schematic diagram of expected mechanisms for functionalization of MWCNT with MEA, DEA, and TEA. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

conventional antibacterial agents like amoxicillin on the CNT structure could enhance the antimicrobial performance.[20] Nanocomposite conjugated with CNT depicted worthy antimicrobial activity against various strains particularly the resistant strain *Staphylococcus aureus*.[21] Meanwhile, in another research in this area, the antimicrobial activity of CNT was improved by functionalization with the metal NPs such as silver and copper NPs.[22]

Despite the toxic nature of CNT, functionalization method could prepare less-toxic material in compared with the pristine one *in vitro* and *in vivo*.[23,24] To develop the antimicrobial activity of CNT, they were functionalized with various functional groups such as metallic NPs and biochemical functionalities.[9,25–28] Despite these materials are more active disinfectants, they showed that are unstable and separate from the main structure, easily.[10]

Meanwhile, the functionalized CNT with biologic groups are more decent and environmental friendly, because of the nature of the functionalities.[29,30] For instance, functionalized CNT with poly-lysine have revealed insignificant damage to the human body and environment, respectively. Amiri et al.[7] and Zardini et al.[9] depicted the antimicrobial property of these amino acids-CNT. They showed functionalized CNT by cationic amino acids have significant antibacterial activity against various bacteria, including *E. coli*, *S. aureus*, and *Salmonella typhimurium*. They suggested the positively charged groups could be responsible for more damage on the membranes of bacteria.

The above-mentioned results demonstrate that the kind of functional groups influences directly on the performance of antibacterial activity.

Here, multiwalled carbon nanotubes (MWCNTs) were first functionalized with mono-, di-, and triethanolamine (MEA, DEA, and TEA) by microwave method, which include a quick and effective process. To prove functionalization, morphological and chemical characterization instruments include Fourier transform infrared (FTIR), thermogravimetric analysis (TGA), and transmission electron microscopy (TEM) were used. Meanwhile, antimicrobial activities of pristine and treated samples (MWCNT-MEA, -DEA, and -TEA) were tested against different bacteria species. To check antimicrobial activity of treated samples as well as pristine MWCNT, four Gram-negative bacteria (*E. coli*, *Klebsiella pneumonia*, *Pseudomonas aeroginosa*, and *S. typhimurium*) and four Gram-positive bacteria (*Bacillus subtilis*, *S. aureus*, *Bacillus cereus*, and *Streptococcus pneumonia*) were investigated by minimal inhibitory concentration (MIC) and radial diffusion assay (RDA).

EXPERIMENTAL

Materials

All the chemicals were of analytical grade and were used without any further purification. The pristine MWCNT (diameter 10–20 nm, length 5–15 μm, purity $>$ 95 wt %, and ash $<$ 1.5 wt %) were purchased from Shenzhen Port Co. MEA, DEA, TEA, *N*,*N*-dimethylformamide (DMF), nitric acid, sulfuric acid, hydrochloric acid, and aluminum chloride were obtained from Merck.

Functionalization of MWCNT

The major approaches for functionalization of CNT include: (1) carboxylation of CNT following by amidation and/or esterification and (2) direct attachment of functional groups to the pristine CNT.[31–33] For increasing the probability of functionalization, we provide a condition for coexistence of both aforesaid approaches. Anticipated mechanisms for functionalization of MWCNT with MEA, DEA, and TEA (MWCNT-MEA, -DEA, and -TEA) are shown in Figure 1.

Accordingly, the pristine MWCNT (150 mg) were added to a (3:1, v/v) mixture of H_2SO_4/HNO_3 (60 mL) and stirred for 12 h by a magnetic stirrer at room temperature. The mixture was then vacuum-filtered through a (0.2 μm pore size) polytetrafluoroethylene (PTFE) membrane and washed with distilled water until the pH of the filtrate reached 6.5. The sample was dried in an oven (50°C) for 24 h. The resultant MWCNT-COOH (150 mg) along with an excess of $AlCl_3$ (1 g) were grounded in an agate mortar for about 5 min. This mixture was then transferred into a vessel filled with 30 mL DMF as well as 15 mL of intended ethanolamine (EA) and sonicated for 10 min, until a visually homogeneous suspension was obtained. Following the addition of concentrated HCl (0.5 mL), the suspension was irradiated by a homemade microwave oven (LG-4284TCR) equipped with a proportional-integral-derivative (PID) controller for 15 min. The microwave power and temperature was set to 800 W and 100°C, respectively. This PID controller only adjusted the temperature. This microwave turn on with power of 800 W and when the temperature reached to 100°C, the heating of vessel were concluded. Subsequently, the reaction mixture was filtered through a PTFE membrane and thoroughly washed with DMF and abundant deionized water to remove the unreacted excess reagents. Finally, the resulting black solid was dried overnight at 50°C and functionalized MWCNT were obtained.

Characterizations

FTIR spectroscopy (Thermo-Nicolet Avatar 370) and TGA (TGA-50 Shimadzu) were used to study the type and amount of functional groups on the surface of MWCNT. The FTIR spectra evaluated in the range of 4000–400 cm^{-1} using KBr pellets and TGA experiments were carried out with a heating rate of 10°C/min under air atmosphere. For morphological analysis, functionalized MWCNT were dispersed in ethanol by an ultrasonic bath (10 min) and deposited on a holey carbon copper grid. After air-drying, samples were investigated by a TEM (LEO 912 AB electron microscope).

RDA and MIC

Eight bacteria (four Gram positive and four Gram negative) were used for antimicrobial assay that include *E. coli* PTCC2433, *K. pneumonia* PTCC4231, *P. aeroginosa* PTCC2834, *S. typhimurium* PTCC4833, *B. subtilis* PTCC4533, *S. aureus* PTCC1442, *B. cereus* PTCC1435, and *S. pneumonia* PTCC 1235. The antimicrobial activity of pristine and functionalized MWCNT was done by two methods: RDA and MIC. Both methods were done according to ultrasensitive methods described in our previous articles with slight modification.[9,34,35] In RDA, all of the bacteria were grown in tryptic soy broth (TSB) at 37°C overnight. The 50 mL of the mentioned culture was added to 50 mL of fresh TSB and incubated for an additional 2.5 h at 37°C. Bacterial cells were centrifuged at 900*g* for 10 min, washed once with cold 10 m*M* sodium phosphate buffer (NAPB) and resuspended in 10 mL of cold NAPB. According to absorbance at 630 nm, 4 × 106 CFU of bacteria was added to 6 mL of underlayer agar broth (0.03% TSB and 1% agarose) and the agar was poured into a Petri-dish. Samples were added directly to 3-mm diameter wells that were made on the culture with puncher. After incubation for 3 h, the enriched medium (6% TSB and 1% agarose) was poured on under layer culture and incubated overnight at 37°C. The antimicrobial activities were assayed by observing the suppression of bacterial growth around the 3-mm diameter wells.

MIC was determined to quantify antimicrobial activity. Stock serial dilutions of 0.01–1 mg/mL of the samples were prepared and 20 μL of them were added to a liquid media culture containing 10^6 CFU/mL of bacteria, which was poured into a micro plate. The ultimate concentrations of the samples were 1–100 μg/mL. The micro plate was incubated at 37°C for 18 h. After this time, the absorbance was read at 630 nm with enzyme-linked immunosorbent assay reader. The MIC was defined the lowest concentration that the bacterial growth was completely inhibited.[7,9,35]

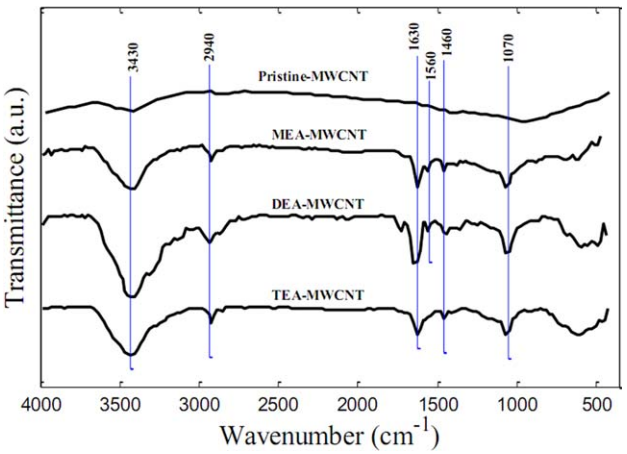

FIGURE 2. FTIR spectra of pristine MWCNT, MWCNT-MEA, -DEA, and -TEA. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

RESULTS AND DISCUSSION

Functionality analysis

FTIR spectroscopy. The FTIR spectra of pristine MWCNT as well as MWCNT-MEA, -DEA, and -TEA are shown in Figure 2 and the relevant spectra analysis is listed in Table I. Contrary to pristine MWCNT, the treated samples showed clear evidence of the attached functionalities. Because no hydrogen atoms directly bonded to the amine group of TEA, the functionalization of MWCNT by TEA is not carried out by

TABLE I. The Spectral Analysis of FTIR Peaks

Peak Wavenumber (cm^{-1})	Spectral Analysis
3500–3350	O–H stretching
3430	N–H stretching
2940	C–H stretching
1630	C=O stretching
1560	N–H bending
1460	C–H_2 bending
1070	C–O stretching

amidation reaction.[36] This confirmed by the absence of N–H bond in FTIR spectra of MWCNT-TEA. Also, the observed OH bond in case of MWCNT-MEA reveals that the esterification is not the only functionalization mechanism there. Therefore, it may be concluded that different EAs follow different mechanisms for reaction with MWCNT and at least two mechanisms coexist in all functionalization procedures.

Thermogravimetric analysis. The amount of the attached organic moieties in the functionalized samples can be determined by TGA. Figure 3(A) shows the TGA curve of the pristine MWCNT in comparison with MWCNT-MEA, -DEA, and -TEA. The TGA traces of treated samples were exhibited considerable weight loss between 140 and 500°C, corresponding to the decomposition of EAs attached to MWCNT, while pristine MWCNT give hardly any weight loss under identical conditions.[34,37,38]

It can be seen from Figure 3(A) that the weight losses of the treated samples are in the sequence: MWCNT-MEA ∼ MWCNT-DEA < MWCNT-TEA. Accordingly, MWCNT-TEA has a lower functionalization yield that may be due to the steric hindrance of large molecules[34,36] and/or this fact that TEA cannot react with MWCNT by amidation reaction.

Figure 3(B) shows the TGA curve of the MWCNT-MEA with various degree of functionalization. The sharp mass loss observed in the temperature range of 100–250 °C could be attributed to the MEA groups. The TGA curves of various functionalized samples show a relatively different mass loss at mentioned temperature range. On the basis of this mass loss, the degree of functionalization of CNTs are different. The resulted curves in Figure 3(B) are shown mass loss percentage of functional groups of 15.25, 27.44, and 39.73 for MWCNT-MEA-1, MWCNT- MEA-2, and MWCNT-MEA-3, respectively.

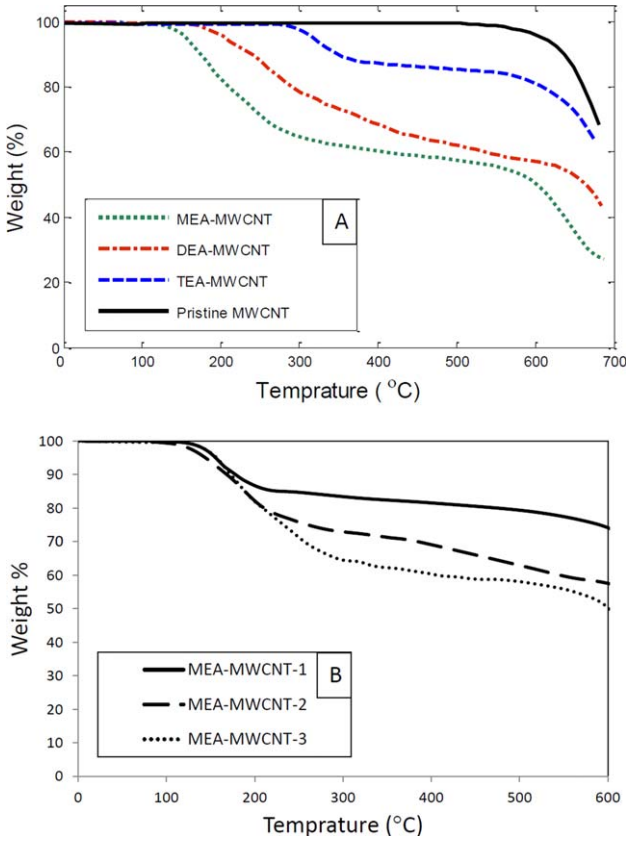

FIGURE 3. TGA curves of (A) the pristine MWCNT, MWCNT-MEA, -DEA, and -TEA and (B) the various treated MWCNT with MEA. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Transmission electron microscopy. As we know, the chemical functionalization disrupts the extended π-conjugation of nanotubes.[38,39] Comparing the TEM images of pristine and functionalized MWCNT (Fig. 4), it is observed that after functionalization, the surface roughness increase as expected. Both the oxidation and microwave radiation may

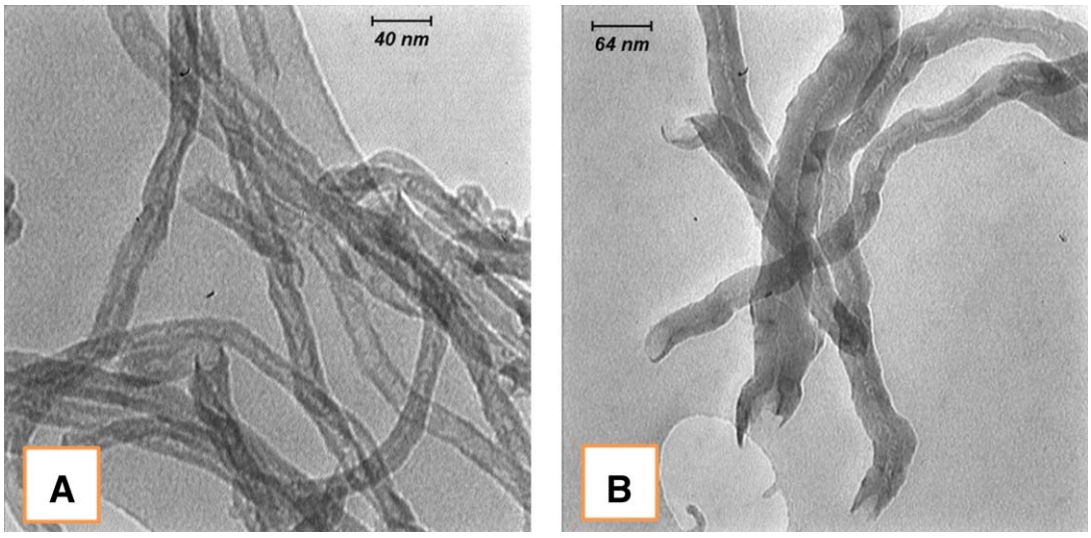

FIGURE 4. TEM images of (A) MWCNT-MEA and (B) MWCNT-TEA. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

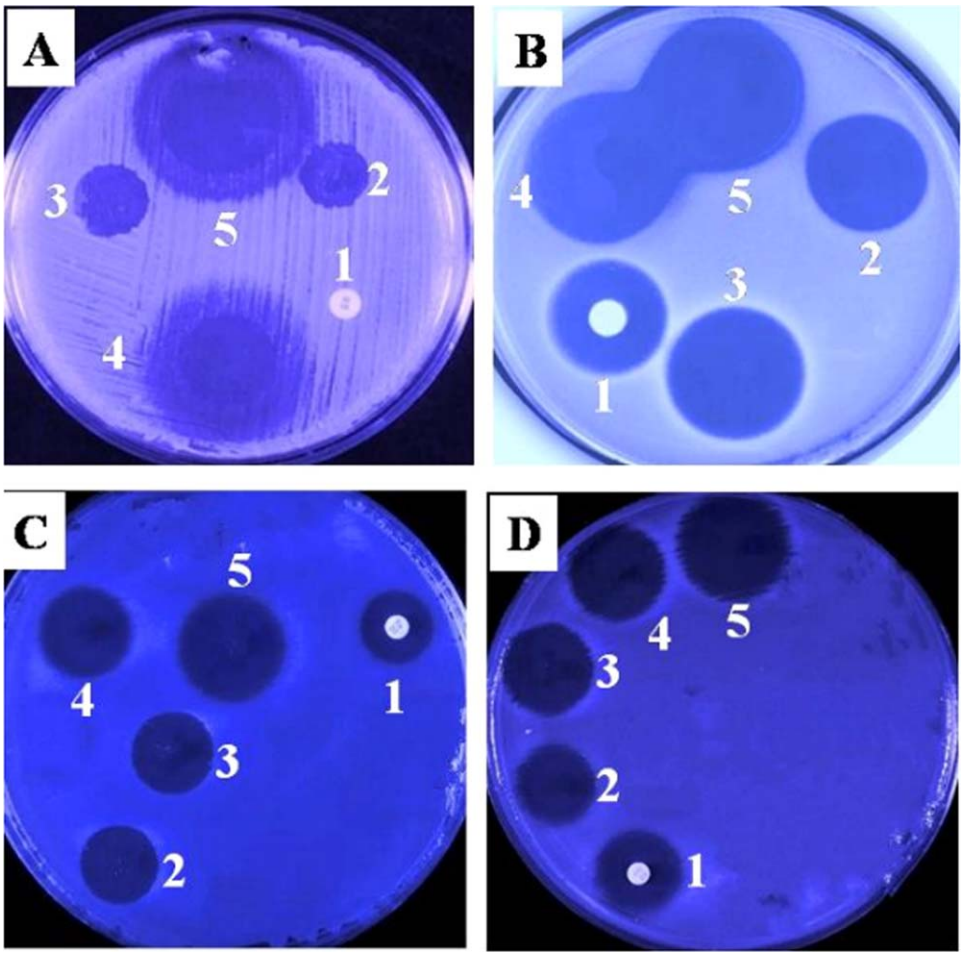

FIGURE 5. The antimicrobial activity of pristine MWCNT (2), MWCNT-MEA (3), MWCNT-DEA (4), and MWCNT-TEA (5) against Gram-negative bacteria: *Escherichia coli* (A), *Klebsiella pneumonia* (B), *Pseudomonas aeroginosa* (C), and *Salmonella typhimurium* (D) using the RDA method (inhibition zone). *Escherichia coli* is the antibiotic resistant bacterium. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

affect the partial damage to the graphitic structure of MWCNT.[40]

Antimicrobial assay

The results of the antimicrobial assay of samples are summarized in Figures 5 and 6 and Table II. According to these data, all of functionalized nanostructures in comparison with pristine MWCNT have higher antimicrobial activity against eight tested bacterial strains. The diameters of growth inhibition zone of samples (10μg/mL) were larger than neomycin (10 μg/mL; Figs. 5 and 6). Two antibiotic resistant bacteria (*E. coli* and *S. aureus*) were also used in this study. All samples have potent inhibitory activity against these two resistant bacteria. In between, the highest antimicrobial activity was observed from MWCNT-TEA, so that this sample completely inhibits bacterial growth in the range of 4.22–14.22 μg/mL (Table II). Among the tested microorganisms, the Gram-negative bacteria are more sensitive than Gram-positive bacteria. This result was observed in functionalized MWCNT with amino acids at previous articles, too.[7,9] As shown in Figure 5 and Table II, the pristine MWCNT, MWCNT-MEA, MWCNT-DEA, and MWCNT-TEA inhibit Gram-negative bacterial growth in the range of 8.25–9.12, 5.23–8.65, 3.36–8.23, and 2.87–6.95, respectively. The higher ability of the MWCNT-TEA, MWCNT-DEA, and MWCNT-MEA to kill bacteria may be because of adding amine groups to MEA and DEA. This event could attribute to the increasing positive charge that initiated increase in the interaction with negatively charged membranes of the bacteria.[9,27,41,42]

It was found that the antibacterial activity of the functionalized nanotubes (with MEA, DEA, and TEA) is better than the native one, and increases by increasing the amount of introduced amine groups. Similarity, this data was obtained in another article with chitosan chemically modified by DEA.[43] Previous studies indicated that amine compounds have a wider spectrum of antimicrobial activity.[44–46] The increase in the amino group substitution on the nanotubes increases the positively cationic nature of this structure which lead to increase the chance of an interaction between the nanotubes and the negative charge on the cell walls of the microorganisms.[47] On the other hand, the

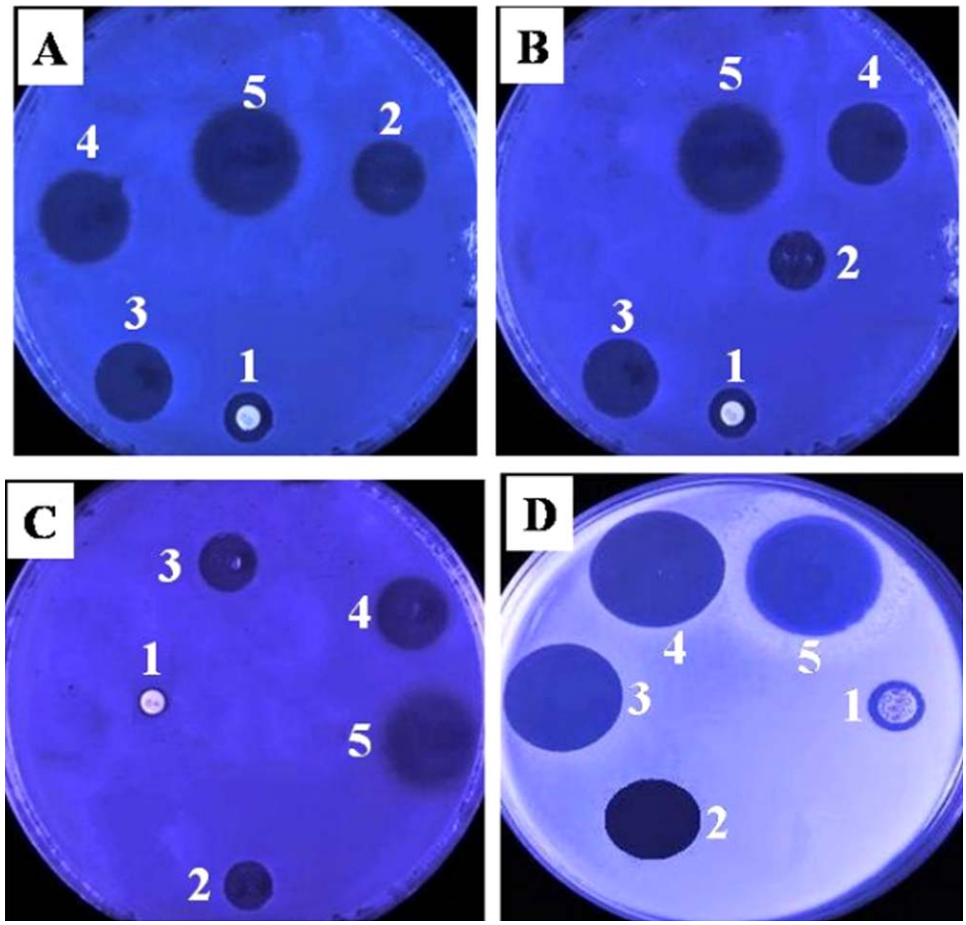

FIGURE 6. The antimicrobial activity of pristine MWCNT (2), MWCNT-MEA (3), MWCNT-DEA (4), and MWCNT-TEA (5) against Gram-positive bacteria: *Bacillus cereus* (A), *Bacillus subtilis* (B), *Staphylococcus aureus* (C), and *S. pneumonia* (D) using the RDA method (inhibition zone). *Staphylococcus aureus* is the antibiotic resistant bacterium. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

interaction of the amine groups of functionalized nanotubes with the cell wall decrease its selective permeability which leads to leakage of the intracellular substance, such as protein, amino acids and glucose dehydrogenase and so, functionalized nanotubes inhibit the normal metabolism of microorganisms and finally lead to death of this cell.[48–50]

To study the influence of functional groups on antimicrobial activity, first three functionalized MWCNT with various degree of functionalization were prepared. To illustrate various degree of functionalization, Figure 3(B) were used by aid of TGA. The resulted curves in Figure 3(B) are shown mass loss percentage of functional groups of 15.25, 27.44,

TABLE II. The MIC Values of Pristine and Functionalized MWCNT Against Various Bacteria

	MIC (μg/mL)[a]			
Microorganism	Pristine MWCNT	MWCNT-MEA	MWCNT-DEA	MWCNT-TEA
Gram negative				
Escherichia coli	8.25 ± 0.15	6.32 ± 0.12	4.23 ± 0.21	4.22 ± 0.09
Klebsiella pneumonia	6.65 ± 0.14	5.38 ± 0.15	3.36 ± 0.08	2.87 ± 0.11
Pseudomonas aeroginosa	6.12 ± 0.16	5.23 ± 0.11	5.15 ± 0.1	3.12 ± 0.16
Salmonella typhimurium	9.12 ± 0.17	8.65 ± 0.12	8.23 ± 0.09	6.95 ± 0.17
Gram positive				
Bacillus cereus	14.32 ± 0.15	11.86 ± 0.13	9.32 ± 0.18	8.2 ± 0.19
Bacillus subtilis	14.65 ± 0.18	12.85 ± 0.17	9.63 ± 0.15	7.13 ± 0.13
Staphylococcus aureus	36.41 ± 0.16	28.12 ± 0.09	20.45 ± 0.14	14.22 ± 0.17
Streptococcus pneumonia	16.5 ± 0.11	10.36 ± 0.08	9.10 ± 0.21	7.56 ± 0.16

[a]Each experiment was repeated at least three times.

TABLE III. The MIC Values of MWCNT-MEA Against Various Bacteria in Different Mass Percentage of Functional Groups of 15.25, 27.44, and 39.73

	MIC (μg/mL)		
Microorganism	MWCNT-MEA-1 (15.25)	MWCNT-MEA-2 (27.44)	MWCNT-MEA-3 (39.73)
Klebsiella pneumonia	5.4 ± 0.12	5.28 ± 0.08	4.45 ± 0.09
Bacillus subtilis	114.63 ± 0.15	14.61 ± 0.1	14.54 ± 0.11

TABLE IV. The Ratio of Percentage of Functional Groups per Microbial Toxicity

Type of Treated Sample	Functional Groups Percentage on the MWCNT Structure	MIC Values of Treated Samples Against *Pseudomonas aeroginosa*	MIC Values/Percentage of Functional Groups for *Pseudomonas aeroginosa*	MIC Values of Treated Samples Against *Bacillus cereus*	MIC Values/ Percentage of Functional Groups for *Bacillus cereus*
MEA-MWCNT	39.73	5.23	0.1316	11.86	0.2985
DEA-MWCNT	28.11	5.15	0.1832	9.32	0.3316
TEA-MWCNT	12.46	3.12	0.2504	8.2	0.6581

and 39.73 for MWCNT-MEA-1, MWCNT-MEA-2, and MWCNT-MEA-3 respectively. Then, the antimicrobial activities of the treated MWCNT with various amounts of functional groups were investigated. Consist with our result [Fig. 3(B) and Table III], reasonable variation are visible on antimicrobial activity of treated sample with MEA. The results show that higher concentration of MEA means higher antimicrobial activity and/or performance. To more confidence at work, this experiment investigated on a Gram-negative bacterium and a Gram-positive bacterium.

Meanwhile, the quantitative tests (MIC results) show similar results and again confirmed the qualitative results. Finally, the increased antimicrobial activity of functionalized MWCNT could attribute to the functional groups. Different MIC results of various treated samples with MEA in mass percentage of 15.25, 27.44, and 39.73 verify this claim against various bacteria.

In order to investigate the effect of functional groups, Table IV is reported the amount of the functional groups on MWCNT structure and antimicrobial activity of corresponded treated sample. If we assume that the antimicrobial activity of MWCNT is similar for all samples, there are a relation between functional groups and antimicrobial activity that show in Table IV. In agreement with reported results in Table IV, the ratio of MIC values per percentage of functional groups in functionalized sample depict that the sequence of antimicrobial activity was as follow:

MWCNT-TEA > MWCNT-DEA > MWCNT-MEA.

This trend is approximately similar for other mentioned bacteria. The abovementioned sequence shows that microbial toxicity of TEA is significantly larger than those of DEA and MEA groups.

CONCLUSIONS

Here, the effective technique of microwave radiation for MWCNT functionalized with MEA, DEA, and TEA were used. MWCNT surface functionalization was confirmed by FTIR, TGA, and TEM results. Eight bacteria species were selected to measure the antimicrobial activity of pristine and functionalized MWCNT. The based on MIC and RDA results show that the antimicrobial activity of functionalized MWCNT was better than pristine MWCNT. In between, antimicrobial activity of MWCNT-TEA in all bacteria species is slightly higher than that of MWCNT-DEA and MWCNT-MEA, respectively. Of course, values of pristine and functionalized MWCNT against Gram-positive bacteria were less compared to Gram-negative bacteria. Based on the results, it seems that EA groups could play an important role in antimicrobial activity of MWCNT. Amino groups in the chains of EA have a greater spectrum of antimicrobial activity because of positively cationic nature of this structure, which lead to increase the chance of an interaction between the nanotubes and the negative charge on the cell walls of the microorganisms. So, functionalized nanotubes with such functions could efficiently inhibit the normal metabolism of microorganisms and finally lead to death of this cell.

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