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The Effect of Temperature and Acidity on Antimicrobial Activities of Pristine MWCNTs and MWCNTs-Arg

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Abstract

Carbon nanotubes (CNTs) have very promising applications for inhibition of microbial growth. The aim of this study is investigation and comparison of the effect of temperature and acidity on antimicrobial activities of pristine Multiwalled Carbon nanotubes (MWCNTs) and Multiwalled Carbon nanotubes-Arginine (MWCNTs-Arg). Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were calculated in range of temperature (25, 37 and 42 ºC) and pH (4.2, 7.2, and 10) on Staphylococcus aureus. The results approved that pristine and functionalized MWCNTs have broad-spectrum antimicrobial activities against examined pathogen. Between these agents, MWCNTs-Arg and pristine MWCNTs and have the highest inhibitory activity on microbial growth, respectively. The MBC value of MWCNTs was improved by amino acid functionalization. The optimal pH for antimicrobial activity of pristine MWCNTs and MWCNTs-Arg are 4.2 and 7.2 and optimal temperatures are 42 ºC and 42 ºC, respectively. There is no change on optimal temperature of MWCNTs by this functionalization, but functionalization of MWCNTs by Arg enhanced its antimicrobial activity and led to change of optimal pH of MWCNTs for antimicrobial activity. This changes lead to suitable improvement of antimicrobial activity in neutral and biological pH.

Keywords: *Antimicrobial, Arginine, MWCNTs, PH, Temperature.*

1. INRODUCTION

Carbon nanotubes (CNTs) are one of nanostructures with good chemical properties for use in different fields of sciences [1]. CNTs are especially used as industrial and biomedical agent [2, 3]. The one of important properties of these compounds is antimicrobial activities against bacteria, viruses and fungal pathogens [4, 5]. CNTs are divided to 2 categories: single-walled nanotubes (SWNTs) and multi-walled nanotubes (MWNTs) and both categories have antimicrobial activities [6]. In between, the antimicrobial activity of SWCNTs is more than MWCNTs. The toxicity of SWCNTs is also more than MWCNTs [7]. The increase of antimicrobial activity of CNTs is important for application of these nanostructures as antimicrobial agents [8]. For use of these compounds, it's also necessary to decrease their side effects especially hemolytic activity. Functionalization of MWCNTs by different natural and synthetic compounds is the best methods for improvement of their biological properties [9, 10]. Many researchers functionalized the MWCNTs by different functional groups and investigated their chemical and biological properties. These functionalizations have good and bad effects on chemical and biological properties and activities of pristine MWCNTs [11-13]. In best proposed functionalization method, the suitable biological activities were improved and also side effects of these nanostructures such as hemolytic activity were decreased [14, 15]. The most important mechanism for biological activity of MWCNTs is physical interaction with cell membrane and inducement pore in membrane and disruption its integrity [4, 16]. So, the functionalization of MWCNTs must enhance their physical interaction with bacterial cell membranes and decrease their physical interaction with mammalian cell membranes. So, functionalization must due to selective nitration. In our previous study, we showed that the functionalization of MWCNTs with Arginine can improve their antimicrobial activities [17]. The environ-mental condition including temperature and pH, have important effects on activity of nanostructures. So, in this study, the effects of these conditions were assessed on antimicrobial activity of MWCNTs-Arg and were comprised with pristine MWCNTs.

2. EXPERIMENTAL

2.1. Functionalization of MWCNTs

The functionalization of MWCNTs with Arg was done according to our previous studies. Briefly, MWCNTs-COOH was produced by mixture of MWCNTs, HNO3, and H2SO4 and then, these agents were functionalized with Arg by industrial microwave (Milestone MicroSYNTH programmable microwave system).

2.2. Characterization of Nanostructures

For characterization of powdered MWCNT samples, Raman spectroscopy (Thermo Nicolet Almega Dispersive), and transmission electron microscopy (TEM, LEO 912 AB electron microscope) was used. Similar to previous study, the Raman Spectrometer system included an optical microscope that was used for fine locating. To avoid thermal decomposition, the laser power was adjusted as low as 30 mW. TEM samples were also prepared by suspending the MWCNTs in ethanol and dropping the suspension on a carbon-coated copper grid.

2.3. Bacterial Strains

In this study, one-gram positive bacteria (Staphylococcus aureus) were used. This bacterium was antibiotic resistant strain isolated from clinical specimens in Shahid Sadoughi hospital, Yazd, Iran.

2.4. Antibacterial Susceptibility Test

The minimal Inhibitory Concentration (MIC) was assessed for each nanostructure in this study. This value was acquired according to our previous studies. The

serial dilution of any nanostructure was prepared. These concentrations of nano-structures were added to solutions containing 106 CFU/ml of bacterium (Staphylococcus aureus) and then, they were poured into wells of microplate.

The final concentrations that used for evaluation MIC were: 5, 10, 20, 40, 60, 100, 200, 300, 400 and 500 µg/ml. the plate was incubated at 37 °C. After 18 hours, the absorbance for each well was read at 630 nm using an enzyme-linked immunosorbent assay (ELISA) reader and the results were compared with control samples. The MIC was defined the minimum sample concentration that bacterium has no growth in it. For calculation of MBC, all bacteria were incubated with serial different concen-tration of nanostructures. After 24 hour, 100µl of bacterium solution were cultivated on nutrient agar and then, plates were incubated for 48 hour at 37ºC. The decrease of bacterial growth was compris-ed with control. In MBC calculation, 30 µL of bacteria suspension (first well of microplate that showed no bacteria growth) was inoculated on to agar plate and then, was incubated for an additional 24 h at 37 °C. The MBC is defined as the lowest concentration of antimicrobial that will prevent the growth of an organism after subculture on to antibiotic free media. In this section, the standard I nutrient broth and nutrient agar media (Merck company) were used.

2.5. Effects of pH on Antimicrobial Activity Antibacterial susceptibility test

The effect of pH on antimicrobial activity of nanostructures was investigated by using different culture media with different pH (4.2, 7.2, and 10). All bacteria were treated with same concentration of pristine MWCNTs, Ag nanoparticles and Ag-MWCNTs. After 24-hour incubation, the absorbance for each well was read at 630 nm using an enzyme-linked immune-sorbent assay (ELISA) reader and the results were compared with control samples and growth curves was drawn.

2.6. Effects of Temperature on Antimicrobial Activity Antibacterial susceptibility test

For assessment of temperature effect, bacteria were treated with same concentration of pristine MWCNTs, Ag nanoparticles and Ag-MWCNTs at different temperatures (20, 37 and 42 ºC). After 24-hour incubation, the absorbance for each well was read at 630 nm using an enzyme-linked immunosorbent assay (ELISA) reader and the results were compared with control samples and growth curves was drawn.

3. RESULTS

3.1. Characterization Results

The FTIR, and Raman results of pristine and Arg-functionalized MWCNT are shown in Figure 1. From FTIR spectra in Figure 1a, in contrast with pristine MWCNTs, the treated MWCNT samples showed clear evidence of the desired functionalities. The detailed list of peaks and their assigned groups are provided in Table 1. It was observed that the carboxyl groups had peaks that were considerably sharper than the peaks of other groups. It is speculated that these sharp peaks resulted from the oxidation step by nitric acid or from the lysine and arginine groups that were attached to the surfaces of the MWCNTs. The N−H groups that were observed on the functionalized samples could indicate that amidation reactions occurred between the amino groups of arginine/lysine and the carboxyl groups on the surfaces of the MWCNTs.

Figure 1b shows the Raman spectra of pristine and Arg-functionalized MWCNT. The Raman spectra of both pristine and functionalized MWCNTs display the D and G bands at 1351 and 1588 cm^{-1}, respectively. The intensity ratio of these bands (ID/IG) is the ratio of amorphous/disordered carbon (sp^3) relative to graphitic carbon (sp^2). In the

functionalization studies, a higher I_D/I_G indicates a greater extent of C=C rupture, implying higher covalent functionalization on the surfaces of the CNTs. As could be seen, I_D/I_G of the MWCNTs-Arg was larger than that of pristine MWCNTs, which implies the successful functionalization of the graphitic walls of the MWCNTs.

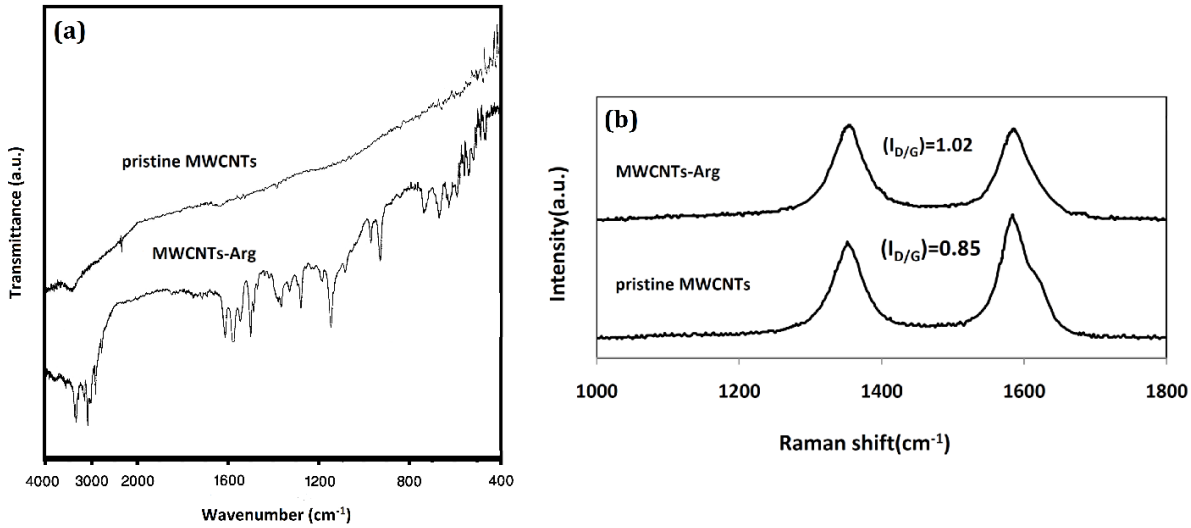

Figure 1. *The FTIR (a), and Raman (b) results of pristine and Arg-functionalized MWCNT.*

Table 1. *Fourier transform infrared interpretation of the pristine and functionalized MWCNTs.*

Type of functionalized MWCNTs	Peak (cm^{-1})	Interpretation
Pristine MWCNTs	2336	Stretching of CNTs backbone
Arginine-treated MWCNTs	3363	–OH Stretching vibration
	3100-3200	–NH stretching vibration of primary amine / Symmetrical –NH stretching vibration (Amide bond)
	2851	–C–H stretching vibration
	1679	–C=O stretching vibration (amide bond)
	1615	–C=O stretching vibration
	1572	–NH bending vibration of primary amine
	1465	$–CH_2$ bending vibration
	1330	–C–N stretching vibration (amide bond)
	1142	C–O stretching vibration

3.2. Antibacterial Susceptibility Test

The results of determination of MIC and MBC were summarized in Table 2. From Table 2, the MIC value for pristine MWCNTs was 14.8 µg/mL against *S. aureus*. Note that this value is 6.9 µg/mL for MWCNTs-Arg for *S. aureus*. Also, MBC for *aureus* was reported to be 31.1 and 13.5 µg/mL for pristine MWCNTs and MWCNTs-Arg, respectively (Table 2).

Table 2. *Minimum inhibitory (MIC) and bactericidal (MBC) concentrations of MWCNTs and MWCNTs-Arg on S.aureus*

	MIC	MBC
MWCNTs	14.8	31.1
MWCNTs-Arg	6.9	13.5

3.3. The Effect of pH

The effect of pH on the antimicrobial activity of pristine and functionalized MWCNTs in the presence of different concentration of nanostructures and different pH (4 to 10) was acquired.

According to the growth curves, both bacteria can grow in all pH except 3.5. So, for investigation of pH effect, the all tests were done in three different acidic, neutral and basic pH (4.2, 7.2, and 10). In order to interpretation of data obtained from different pH effect on the antimicrobial activity of MWCNTs and MWCNTs-Arg, the percentage of growth inhibition was investigated in the pH range in the presence of various concentrations of nanostructures. So, the effect of pH on antibacterial activity of nanostructures was analyzed by comparing the percent inhibition of bacterial growth in different conditions. The results were summarized in Table 3,4, and 5. Based on this table, the antimicrobial activity of MWCNTs increase by enhancement of pH and Vice versa, the decrease of pH to acidic situation leads to increase of antimicrobial activity of MWCNTs-Arg. The optimal pH for antimicrobial activity of MWCNTs and MWCNTs-Arg is 7.1 and 5.2, respectively.

3.4. The Effect of Temperature

The effect of temperature on the antimicrobial activity of pristine and functionalized MWCNTs in the presence of different concentration of nanostructures and different temperature (20, 37 and 42 ºC) was acquired. In order to interpretation of data obtained from different temperature effect on the antimicrobial activity of MWCNTs and MWCNTs-Arg, the percentage of growth inhibition was investigated in the temperature range in the presence of various concentrations of nanostructures. So, the effect of temperature on antibacterial activity of nanostructures was analyzed by comparing the percent inhibition of bacterial growth in different conditions. The results were summarized in Table 3,4, and 5. Based on this table, the antimicrobial activity of MWCNTs and MWCNTs-Arg increase by enhancement of temperature. The highest antimicrobial activity of both nanostructures on two bacterial strains is 42 ºC. So, the functionalization of MWCNTs by Arg don't effect on its antimicrobial activity.

Table 3. *The percentage of growth inhibition in different pH at 42 ºC*

Pristine MWCNTs (µg/ml)	**pH**		
	4.2	**7.2**	**10**
5	67	56	31
100	79	75	48
500	100	100	100
MWCNTs-Arg	**pH**		
5	75	89	66
100	86	94	80
500	100	100	100

Table 4. *The percentage of growth inhibition in different pH at 37 ºC*

Pristine MWCNTs (µg/ml)	**Temperature**		
	4.2	**7.2**	**10**
5	59	52	29
100	68	61	45
500	100	100	100
MWCNTs-Arg	**Temperature**		
5	70	88	59
100	81	93	75
500	100	100	100

Table 5. *The percentage of growth inhibition in different pH at 25 ºC*

Pristine MWCNTs (µg/ml)	**pH**		
	4.2	**7.2**	**10**
5	50	48	22
100	60	58	40
500	100	100	100
MWCNTs-Arg	**pH**		
5	70	85	58
100	76	94	71
500	100	100	100

3.5. Response Surface Analysis

For better analysis of the effects of temperature and pH on antimicrobial activities in two examined nanostructures, Response Surface Analysis was done. We calculated mutual effects of variable by general linear model (response versus Nano, concentration, pH, and temperature) (Table 6&7). Based on table 6, all variables have significant mutual effects except nano/temperature and pH/temperature. The adjusted R-sq. for this analysis was 97.14 %. The results of Table 7

showed that the best pH for activity for MWCNTs and MWCNT-Arg was 4.2 and 10, respectively (coefficient of variation for MWCNTs=4.389 and MWCNT-Arg= 2.222). According to coefficient of variation, the results also showed that the best temperature for activity for MWCNTs and MWCNT-Arg was 42 ºC (coefficient of variation for MWCNTs=1.111 and MWCNT-Arg= 1.222).

Table 6. *Analysis of Variance based on General Linear Model*

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Nano	1	3952.7	3952.67	277.15	0.000
concentration	2	15508.0	7754.00	543.69	0.000
pH	2	2262.1	1131.06	79.31	0.000
Temprature	2	364.0	182.00	12.76	0.000
Nano*concentration	2	2001.3	1000.67	70.16	0.000
Nano*pH	2	520.1	260.06	18.23	0.000
Nano*Temprature	2	49.3	24.67	1.73	0.196
concentration*pH	4	1203.2	300.81	21.09	0.000
concentration*Temprature	4	190.0	47.50	3.33	0.024
pH*Temprature	4	23.9	5.97	0.42	0.794
Error	28	399.3	14.26		
Total	53	26474.0			

Table 7. *Coefficient of variation based on General Linear Model: response versus Nano, concentration, pH, Temprature*

Term	Coef	SE Coef	T-Value	P-Value	VIF
Constant	77.000	0.514	149.83	0.000	
Nano					
MWCNT	-8.556	0.514	-16.65	0.000	1.00
MWCNT-Arg	8.556	0.514	16.65	0.000	*
Concentration					
5	-17.333	0.727	-23.85	0.000	1.33
100	-5.667	0.727	-7.80	0.000	1.33
500	23.000	0.727	31.65	0.000	*
pH					
4.2	3.056	0.727	4.20	0.000	1.33
7.2	5.944	0.727	8.18	0.000	1.33
10.0	-9.000	0.727	-12.38	0.000	*
Temprature					
25	-3.000	0.727	-4.13	0.000	1.33
37	-0.333	0.727	-0.46	0.650	1.33
42	3.333	0.727	4.59	0.000	*
Nano*Concentration					
MWCNT 5	-5.111	0.727	-7.03	0.000	1.33
MWCNT 100	-3.444	0.727	-4.74	0.000	1.33
MWCNT 500	8.556	0.727	11.77	0.000	*
MWCNT-Arg 5	5.111	0.727	7.03	0.000	*
MWCNT-Arg 100	3.444	0.727	4.74	0.000	*
MWCNT-Arg 500	-8.556	0.727	-11.77	0.000	*
Nano*pH					
MWCNT 4.2	4.389	0.727	6.04	0.000	1.33
MWCNT 7.2	-2.167	0.727	-2.98	0.006	1.33
MWCNT 10.0	-2.222	0.727	-3.06	0.005	*
MWCNT-Arg 4.2	-4.389	0.727	-6.04	0.000	*
MWCNT-Arg 7.2	2.167	0.727	2.98	0.006	*
MWCNT-Arg 10.0	2.222	0.727	3.06	0.005	*
Nano*Temprature					
MWCNT 25	-1.222	0.727	-1.68	0.104	1.33

MWCNT 37	0.111	0.727	0.15	0.880	1.33
MWCNT 42	1.111	0.727	1.53	0.138	*
MWCNT-Arg 25	1.222	0.727	1.68	0.104	*
MWCNT-Arg 37	-0.111	0.727	-0.15	0.880	*
MWCNT-Arg 42	-1.111	0.727	-1.53	0.138	*
concentration*pH					
5 4.2	2.44	1.03	2.38	0.024	1.78
5 7.2	4.06	1.03	3.95	0.000	1.78
5 10.0	-6.50	1.03	-6.32	0.000	*
100 4.2	0.61	1.03	0.59	0.557	1.78
100 7.2	1.89	1.03	1.84	0.077	1.78
100 10.0	-2.50	1.03	-2.43	0.022	*
500 4.2	-3.06	1.03	-2.97	0.006	*
500 7.2	-5.94	1.03	-5.78	0.000	*
500 10.0	9.00	1.03	8.76	0.000	*
concentration*Temprature					
5 25	-1.17	1.03	-1.14	0.266	1.78
5 37	0.17	1.03	0.16	0.872	1.78
5 42	1.00	1.03	0.97	0.339	*
100 25	-1.83	1.03	-1.78	0.085	1.78
100 37	-0.50	1.03	-0.49	0.630	1.78
100 42	2.33	1.03	2.27	0.031	*
500 25	3.00	1.03	2.92	0.007	*
500 37	0.33	1.03	0.32	0.748	*
500 42	-3.33	1.03	-3.24	0.003	*
pH*Temprature					
4.2 25	-1.06	1.03	-1.03	0.313	1.78
4.2 37	-0.06	1.03	-0.05	0.957	1.78
4.2 42	1.11	1.03	1.08	0.289	*
7.2 25	0.89	1.03	0.86	0.394	1.78
7.2 37	-0.28	1.03	-0.27	0.789	1.78
7.2 42	-0.61	1.03	-0.59	0.557	*
10.0 25	0.17	1.03	0.16	0.872	*
10.0 37	0.33	1.03	0.32	0.748	*
10.0 42	-0.50	1.03	-0.49	0.630	*

4. DISCUSSION

Annually, infectious diseases lead to more than 60, 000 death tolls in US. The big concern in this subject is development of resistant microbial strains [18]. The development of new antimicrobial agents for inhibition this resistant microbe is unavoidable [19, 20]. Carbon nanotubes have potent antimicrobial activity against different microbes (viruses, bacteria, fungi and etc.) [21, 22]. According to previous study, functionalization of CNTs can improve their different characteristics, especially biological properties. The functionalization of MWCNTs by basic amino acids increased their antimicrobial activities, especially against resistant bacterial strains [23-25]. The change of functional groups can change the activity of MWCNTs in different environmental conditions. In our previous study, we showed that functionalization of MWCNTs by Arginine improve their antimicrobial activities. In this paper, the effect of temperature and acidity on antimicrobial activities of pristine MWCNTs and MWCNTs-Arg was investigated. The antibacterial activity of MWCNTs and MWCNTs-Arg was quantitatively evaluated by determination of MIC. MWCNTs were able to inhibit 50% of bacterial growth (MIC) for examined bacterium in concentration of 14.8 µg/ml. This MIC values for MWCNTs-Arg is 6.9 µg/ml. These data were consistent with our previous study [26-28]. The MBC of nanostructures was also calculated. For this, agar dilution method was used. MBC

is the lowest concentration of an antimicrobial agent that is capable to inhibit 99% growth on the plate. The 99% inhibition for S. aureus was observed in concentrations of 31.1 and 13.5 µg/ml in presence of MWCNTs and MWCNTs-Ag, respectively. The effect of different pH and temperature was investigated on antibacterial properties of MWCNTs and MWCNTs-Arg. According to data, the optimal pH for antimicrobial activity of pristine MWCNTs and MWCNTs-Arg are 5.2 and 7.1 and optimal temperatures are 42 ºC and 42 ºC, respectively. The optimal pH for MWCNTs-Arg shifted to neutral pH. The increased ability of the functionalized MWCNTs to kill bacteria is related to an electrostatic interaction with the bacterial membrane [29]. The increase of proton compete with MWCNTs-Arg for electrostatic interaction with the bacterial membrane [17]. The enhancement of pH can remove this competition and so, enhance the antimicrobial activity. According to the evidence provided above, MWCNTs-Arg has cationic groups. Thus, the significantly higher adsorption of MWCNTs-Arg compared to MWCNTs would make the former a much more effective killer of the bacteria. On the other hand, this higher cationic groups lead to more pH effect on MWCNTs-Arg than MWCNTs. The shift of optimal pH to neutral pH is benefit for MWCNTs applications in biological environments. The overall effect of temperature on antimicrobial activities of MWCNTs and MWCNTs-Arg is identical for examined bacterium. The enhancement of temperature leads to increase of antimicrobial activity. So, temperature increases can boost the antimicrobial properties of the nanostructures [30]. The increase of temperature leads to increase of MWCNTs activity due to the more transfer of electrons. The release electrons from the valence band lead to create empty space and reducing agent. These agents reduce water molecules and produce hydroxyl radicals and hydrogen ions [31]. A series of oxidation-reduction pathways occurred that resulted in the production of reactive oxygen species and lead to killing or stopping the growth of bacteria cells [32, 33]. The antibacterial assays of functionalized MWCNTs by Arginine in different pH and temperature suggested that functionalized MWCNTs have significantly greater antibacterial activity against various bacteria. This finding implied that the positively-charged cationic groups of amino acids were very effective of MWCNTs in different pH. On the other hand, the optimal pH for antimicrobial activity of MWCNTs-Arg shifts to neutral pH in comparison with MWCNTs.

5. CONCLUSION

According to acquired data from this study, the optimal pH for MWCNT and MWCNT-Arg is different, so that the optimal pH for antimicrobial activity of MWCNTs-Arg shifts to neutral pH in comparison with MWCNTs. So, by functionalization of MWCNTs with Arginine, the optimal pH for activity is close to neutral and biological pH in addition to increase of antimicrobial activity.

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