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Research Article

Antimicrobial Effect of Multilayered Carbon Nanotubes on Multi-Drug-Resistant *Pseudomonas aeruginosa*

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Abstract

Background: *Pseudomonas aeruginosa* is the primary cause of infection with impaired defense mechanisms. *P. aeruginosa* commonly causes nosocomial infections and is the most common pathogen isolated from patients hospitalized for longer than 1 week. We examined the antimicrobial effect of multilayered carbon nanotubes on multi-drug-resistant.

Materials and Methods: In this research, 20 clinical isolates collected at Motahari Hospital (Tehran, Iran) were compared with the standard (ATCC 27853) and identified as *P. aeruginosa* based on biochemical testing. Conventional disk diffusion assay demonstrated the methicillin resistance of the isolates. Minimal inhibitory concentrations for antibiotics and the multilayer CNTs were determined using the microdilution method. Single-walled CNTs were prepared and their efficacy and potential synergism with antibiotics was assessed.

Results: Synergism against *P. aeruginosa* was evident for methicillin + single-walled CNTs.

Conclusion: The inhibitory effect of single-walled CNTs and methicillin was synergistic against the growth of *P. aeruginosa*.

Keywords: SWCNTs, *Pseudomonas aeruginosa, antibiotic,* Nano antibiotics

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1. Introduction

Pseudomonas aeruginosa is a gram negative bacterium that is environmentally ubiquitous. It can also be an opportunist pathogen.[1] Multi-drug-resistant (MDR) *P. aeruginosa* can be difficult to eradicate using a variety of therapies.[2] The consequence can be severe diseases or worsening of existing conditions, including cystic fibrosis and burns.

P. aeruginosa typically causes infections in immunocompromised individuals, but can be active in immunocompetent individuals. The persistence of *P. aeruginosa* in a variety of habitats hinders treatment,[3] as does its resistance to carbapenem.[4, 5] Carbapenem antibiotic is often the antibiotic of last resort in severe and life threatening infections caused by MDR gram negative bacteria. Understanding the resistance to carbapenem is important.[6] As well, the efficacy of conventional antibiotics can be compromised as a result of their overuse and they can have side effects. Drugs that are more effective with fewer side effects are needed.[7]

In seeking alternatives, the unique properties of nano-sized structures have attracted attention.[8] Nanoparticles with antimicrobial activity can more active, less toxic, and less expensive compared to the antibiotics that are currently used. In addition, nanoparticles administered in smaller doses can have a longer half-life in the body. Biological nanotechnology is one of the most promising fields of basic science and the new material being developed have potential value in medical biology.[9, 10] Carbon nanotubes (CNTs) are the first generation of nanoparticles, which have been commercially available for nearly two decades.[7] Nanoparticles conjugated with conventional antibiotics have many benefits, including minimizing the side effects of the antibiotics and facilitate the binding of antibiotics to the target microorganisms.[11]

Nano-materials alone or conjugated with specific compounds, such as antibiotics, may have potential therapeutic value.[12] Conjugation of nanoparticles with antibiotics may produce a synergistic effect, which can permit lower doses of the antibiotics to be used, minimizing the side effects.[13] In this study, we investigated antimicrobial properties of multilayered CNTs for clinical isolates of MDR *P. aeruginosa*.

2. Materials and Methods

2.1. Clinical isolates

Twenty P. *aeruginosa* isolates were collected from blood, cerebrospinal fluid, urine, wound swabs, urethral swabs, and sputum from patients during treatment at Motahari hospital (Tehran, Iran) in a 3-month period. All isolates were characterized by biochemical assay. *Pseudomonas aeruginosa* (ATCC 27853) was used as the standard strain.

2.2. Screening of antibacterial activity by disk diffusion

The conventional method of agar diffusion was used to assess antibiotic susceptibility. A single colony of each isolate was inoculated into a test tube containing 2 ml LB broth (Oxoid, Basingstoke, UK) and incubated overnight. The culture of each isolate was diluted with sterile distilled water to a McFarland standard turbidity of 0.5. A sterile swab was dipped into each suspension and used to inoculate Mueller-Hinton Agar (Merck, New York, NY, USA). Discs individually containing clindamycin, ciprofloxacin, ofloxacin, and imipenem (Abtek, USA) were placed on the inoculated plates and then incubated for 18 h at 37 °C. The diameters of the resulting inhibition zones (e.g., Figure 1) were measured and the minimum inhibitory concentration (MIC) was determined as detailed by 2015 Clinical & Laboratory Standards Institute criteria.

Figure 1: MIC test for P. *aeruginosa* .

2.3. Preparation of multilayered CNTs

CNT in powder form for MIC testing was purchased as single packs (US Research Co., USA). Specifications and characteristics are summarized in Tables 1 and 2. Multi-walled CNTs (MCNTs) were functionalized using $3H_2SO_4$:$2HNO_3$ solution with 98% and 65% acid concentrations, respectively. The mixture of the acid solution and MWCNTs were kept at 140°C for 4 h, followed by dilution in distilled water for 6 h until the pH reached 7. The diluted solution was filtered through a 0.2 µm filter. The functionalized MWCNTs

were dried in a 60˚C oven for 24 h and characterized using thermogravimetric analysis (TGA) scanning electron microscopy(FEI Quanta 200 ESEM).

Table 1: Specification and characteristics of multi-walled carbon nanotubes (MWCNTs).

Characteristics	Details
MWCNTs	
Purity	> 95 wt% CNTs as determined using TGA and TEM
Carbon content	> 97 wt%
Outside diameter	20-30 nm, as determined using high-resolution TEM and Raman spectroscopy
Inside diameter	5-10 nm
Length	10-30 um, as determined by TEM
Specific surface area	> 110 m^2/g, as determined by Brunauer–Emmett–Teller gas adsorption method
Color	Black
Ash	<1.5 wt%, as determined by TGA
Electrical conductivity	>100 s/cm
Tap density	0.28 g/cm^3
True density	~2.1 g/cm^3
Manufacturing Method	Chemical vapor deposition

Table 2: Physical properties of CNTs.

Material	SWNT	MWNT	Steel
Young's modulus (GPa)	1054	1200	208
Tensile Strength (GPa)	150	150	0,4
Density (g/cm^3)		2,6	7,8
Thermal Conductivity W/m.K	3000		
Electrical Conductivity S/m	$10^5 - 10^7$		

2.4. Broth microdilution determination of MICs

Broth microdilution was used to determine the MIC of imipenem alone and in combination with SWCNTs. To reach an 8 µg/mL concentration, imipenem and functionalized SWCNTs

(f-SWCNTs) was dissolved in dimethyl sulfoxide (DMSO) and distilled water, respectively. These solutions were serially diluted 11 times with Muller Hinton broth (Merck). For detection of DMSO antibacterial activity, DMSO in Muller Hinton broth was serially diluted from 50% to 0.098%. Each isolate was added to duplicate wells to achieve final bacterial concentration of 5×10^5 colony forming units (CFU)/mL, except for the bacteria-free negative control. The enzyme-linked immunosorbent assay plates were incubated

for 18 h at 35°C. The absorbance of each well was determined at 630 nm using a microplate reader (Awareness Technology Inc., Palm City, FL, USA). Muller Hinton broth was the blank. The lowest concentration with any visible growth was considered to be the MIC.

2.5. Evaluating synergistic effect of antibiotic and CNTs

After determining the MIC, the microdilution broth method was used to evaluate the efficacy of each antibacterial agent, including imipenem, and the MWCNTs prior to conjugation.

3. Results

As per the convention for the agar diffusion test, the results were interpreted based on the presence or absence of an inhibitory zone of growth. If the bacteria were susceptible to the antimicrobial agent, a zone of growth inhibition appeared around the disk. The absence of an inhibition zone demonstrated resistance to the particular antibiotic.

A representative scanning electron microscopy view of MWCNTs is shown in Figure 2.

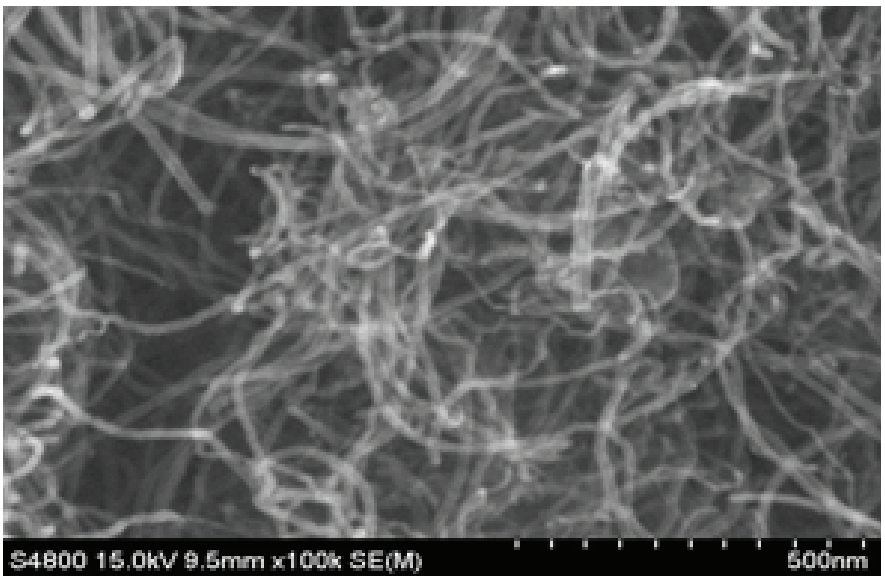

Figure 2: Scanning electron microscopy of MWCNTs.

Of the 20 clinical isolates of *P. aeruginosa*, 17 (85%) were resistant to one or more antibiotics (Table 3). Resistance was highest to clindamycin followed by imipenem. Resistance was lowest for ofloxacin .

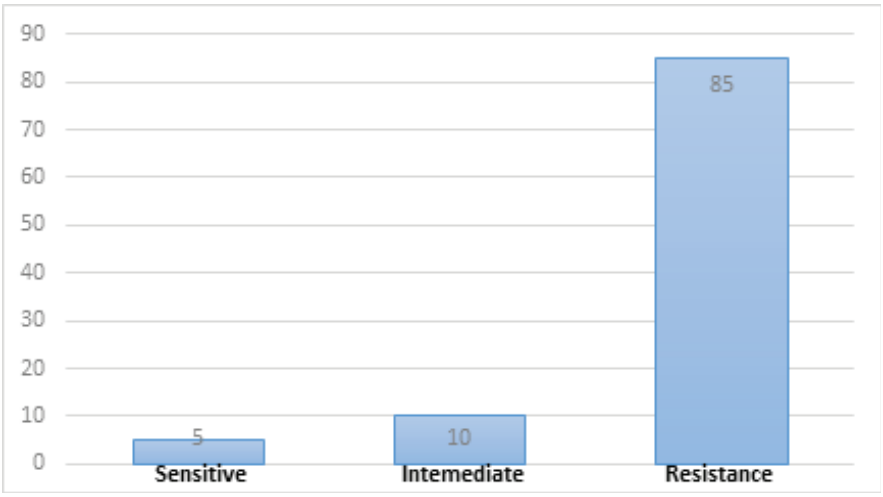

Figure 3: Antibiogram frequency for *P. aeruginosa*.

Antibiogram test results (Figure 4) revealed that resistance was most pronounced to clindamycin and imipenem, and lowest to ofloxacin.

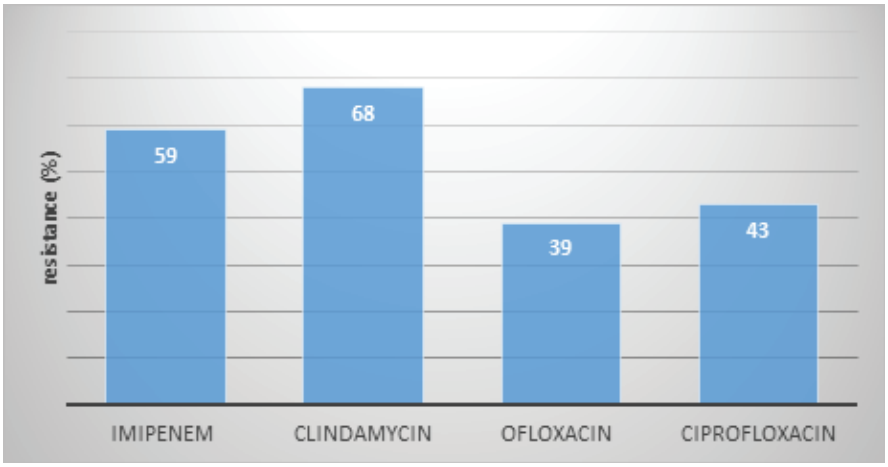

Figure 4: Antibiogram test result showing resistance to antibiotics.

The mean MIC for imipenem, CNTs, and the conjugated combination of both was 32, 16 and 8 µg/mL, respectively, for the clinical samples. The significant difference of the combination ($P<0.005$) indicated a synergistic effect.

4. Discussion

The present comparison of the antimicrobial effect of CNTs and imipenem, which is the antibiotic of last resort for MDR infections caused by gram negative bacteria including *P. aeruginosa*, was prompted by the increased resistance to imipenem and concomitant elevated risk of infection and mortality. The idea behind the study was that CNTs could

be an alternate therapeutic option to conventional antibiotics, with effective antibacterial activity, fewer side effects, and reduced acquisition of antibacterial resistance.[15]

The first data concerning the prowess of MWCNTs were provided a decade ago. The superior antibacterial efficacy and reduced cytotoxicity of MWCNTs compared to SWCNTs were reported. The superior performance against *Escherichia coli* was because of the size.[16]

The present data demonstrates that this antibacterial efficacy extends to clinical isolates of *P. aeruginosa*. The isolates displayed the most pronounced resistance is to clindamycin (68%) followed by imipenem (59%), with the least resistance observed to ofloxacin (39%). The mean MIC of imipenem alone and CNTs alone was 32 µg/mL and 16 µg/mL, respectively. Their combination produced a synergistic response, evident by the mean MIC of 8 µg/mL. The findings support the prior description of the bacteriostatic growth inhibition by silver nanoparticles.[17]

Shrivastav et al. described the use of silver as a therapeutic agent capable of complete growth inhibition of a wide range of gram negative and gram-positive bacteria.[18] Conversely, Anil et al. reported only 25% resistance to amikacin, with more pronounced resistance (75%) to ciprofloxacin.[19] Presently, we observed resistance rates of 68% and 59% to clindamycin and imipenem, respectively. Sandhya et al. described the use of CNTs as a multi-purpose therapeutic agent. The authors reported improved solubility and compatibility of CNT preparations, altered metabolic pathway, and decreased cytotoxicity.[20] Our findings corroborate these prior observations. Niitsuma et al. compared global bacterial resistance to imipenem antibiotics; resistance rates included 14% in Spain, 13.4% in Russia, 12% in Canada, and 8.3% in Japan.[21, 22] The presently observed imipenem resistance rate in Iran is markedly higher (59%), which indicates a dangerous impact on public health.

Haifi et al. analyzed the efficacy of an imipenem-colistin conjugate as a therapeutic combination against *Enterobacter*-resistant to multiple antibiotics. The documented activity of colistin alone and in combination with imipenem against Enterobacter species indicated the therapeutic value of the combination for digestive system infections.[23] Similarly, the combination of imipenem and CNTs was synergistically active against MDR *P. aeruginosa*, indicating that it might be a substitute to carbapenem. Cemal et al. studied the risk of hospital infection due to MDR *P. aeruginosa* with the aim of determining risk factors accelerating the spread of infection. The treatment period and use of carbapenem were important risk factors.[24] Seo et al. recently suggested that the antibacterial properties of MWCNTs can be amplified by conjugation with silver and evaluated

this strategy against *Methylobacterium and Sphingomonas* spp. The synthesized silver-CNTs showed negligible toxicity; however, further studies of its biosafety are necessary before the strategy can be commercialized.[25]

The present and prior findings support the conclusion that MWCNTs are very effective when used along with imipenem, with synergistic activity evident against clinical isolates of *P. aeruginosa*. The strategy may be a valuable replacement for conventional antibiotics in the treatment of *P. aeruginosa* infections.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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