Effect of (ZnO/MWCNTs) Hybrid Concentrations on Microbial Pathogens Removal

Dr. Duha S. Ahmed
Applied Sciences Department, University of Technology/ Baghdad.
Email:duha.saadi@yahoo.com
Ali L. Abed
Nanotechnology Advanced Material Research Center, University of Technology/Baghdad.
Azhar J. Bohan
Nanotechnology Advanced Material Research Center, University of Technology/Baghdad.
Jhan Y. Rbat
Nanotechnology Advanced Material Research Center, University of Technology/Baghdad.

Received on:4/3/2015 & Accepted on:9/7/2015

ABSTRACT
In this study, hybrid material like ZnO coated multi-walled carbon nanotubes (ZnO/MWCNTs) was formation by a multistep chemical procedure and evaluated for their application potentials as an antimicrobial agent for slowing down bacterial growth as bacteriostatic and inactivating pathogenic bacteria as bactericidal. The prepared carbon nanotubes and ZnO nanoparticles were investigated by using X-ray diffraction (XRD) and Scanning Electron Microscopy (SEM). The antimicrobial properties of hybrid material (ZnO/MWCNTs) were tested against Salmonella typhi (S.typhi), Pseudomonas aeruginosa (P. aeruginosa) which are Gram-negative and Staphylococcus aureus (S. aureus) which is Gram-positive by using standard agar dilution (plate count) method.
Keywords: antimicrobial agents**,** ZnO nanoparticles**,** carbon-based nanoparticles**,** photocatalytic materials

تاثير تراكيز ZnO/MWCNTs الهجين على ازالة الميكروبات المرضية

الخلاصة
في هذه الدراسة، تم تصنيع مادة هجينة مثل أوكسيد الزنك المرسبة على الكاربون النانوي المتعدد الجدران ZnO/MWCNTs باستخدام تقنية كيميائية متعددة الخطوات وتقييم امكانية تطبيقها كمادة مضادة لابطاء من نمو الميكروبات وتعطيل الفعل البكتيري. ان عملية تحضير مسحوق الكاربون النانوي المتعدد الجدران و اوكسيد الزنك النانوي تم دراسة خصائصه باستخدام كل من جهاز حيود الاشعة السينية (XRD) و المجهر الالكتروني الماسح (SEM).اما الفعالية المضادة للمادة الهجينة ZnO/MWCNTs فقد تم اختبارها ضد انواع مختلفة من البكتريا المرضية ذات الصبغة السالبة مثل *Salmonella typhi (S.typhi), Pseudomonas aeruginosa* (*P. aeruginosa*) و البكتريا ذات الصبغة الموجبة مثل *Staphylococcus aureus* (*S. aureus*) باستخدام طريقة التخفيف او (طريقة العد)

INTRODUCTION
The increasing resistance of the microorganisms towards antibiotics has been led to serious health problems in the recent years. However, the re-emergence of infectious diseases and the continuous development of antibiotic resistance among a variety of disease-causing bacteria pose a serious threat to public health worldwide .The presence of pathogenic bacteria in the environment is being

https://doi.org/10.30684/etj.2015.116735
2412-0758/University of Technology-Iraq, Baghdad, Iraq

increasingly recognized as a major threat to the health of mankind [1,2]. Many of these organisms are also part of the commensal flora which coexists in a natural equilibrium with the human body. This problem encourages the researchers to study the new agents which can effectively inhibit microbial growth [3,4].
Nanomaterials as the novel drug delivery systems have been also applied to improve the physicochemical and therapeutic effectiveness of the drugs. Hence, new kind of antibacterial agents need to be developed to resist bacterial colonization for longer time. Inorganic antibacterial agents are one such class of antibacterial recently been extensively studied in the last of 5-6 years. Likewise, recent advances in the field of nanotechnology, particularly the ability to prepare highly ordered nanoparticulates of any size and shape, have led to the development of new biocidal agents. Several studies have indicated that nanoparticulate formulations can be used as effective bactericidal materials.
Antimicrobial agents are natural or synthetic compounds that inhibit microbial growth. Various classes of antimicrobial agents are used in the textile industry, most of which are biocides. The advantages of inorganic antibacterial materials are superior to those of organic antibacterial materials in durability, heat resistant, selectivity, and so on [2].
Many heavy metals and metal oxides either in their free state, or in compounds at very low concentrations, are toxic to microbes. These inorganic materials kill bacteria through various mechanisms, such as by binding to intracellular proteins and inactivating them, generation of reactive oxygen species and via directs damage to cell walls. Zinc oxide (ZnO), copper oxide (CuO), magnesium oxide (MgO), titanium dioxide (TiO_2) and silver (Ag) are some of the most commonly used inorganic materials in the fabrication of antimicrobial coatings [5].
Comparing with organic agents, the new inorganic antibacterial agents have more advantages such as broad spectrum, durability, safety, and so on. Because of their good prospects in applications, inorganic antibacterial agents have been widely investigated [6].
Among the metal oxide, ZnO is one of the interesting photocatalytic materials. (ZnO) nanostructures have become the focus of considerable research due to their low cost, easy availability, biocompatibility and possibility of performing surface modifications with different functional groups. They possess unique chemical and physical properties like intensive ultraviolet absorption or antimicrobial activity in the pH range of 7–8 even in the absence of light, therefore are widely used for applications such as optical devices [7,8] and antimicrobials [9]. In particular, these nanostructures are considered to be non-toxic and recent studies have reported that they do not cause any damage to the DNA of human cells [10]. Concerning the photocatalytic property, it was also shown that ZnO-coated MWCNTs exhibited a significantly better photocatalytic activity than bulk ZnO or the mechanical mixture of ZnO and MWCNTs [11,12].
Many researchers are trying to improve and modify MWCNTs walls to achieve high dispersion of metal nanoparticles on the MWCNTs surface. In order to obtain the physical properties of nanoparticles, there should be sufficient connections between nanoparticles and nanotubes. Therefore, Functionaliztion MWCNTs surface with desired functional groups by chemical treatments is a good strategy to get well-dispersed catalyst nanoparticles on MWCNTs. Usually, the chemical oxidation processes, functionalize the surface of MWCNTs. It leads to the formation of some active groups like -OH, -C=O, -C-O, and COOH. They can act as the location for the

formation of the ions of nanoparticles. Therefore, researchers try to find an appropriate procedure for synthesizing and activating the surface of MWCNTs [13].
In present review, the formation of ZnO/MWCNTs hybrid by a multistep procedure was prepared. Functionaliztion F-MWCNTs were prepared by vacuum filtration from oxidized multi walled carbon nanotubes. Zinc acetate dihydrate was used as zinc source and ethanol used as solvent. Then, study the antimicrobial activity of various concentrations of ZnO/MWCNTs hybrid by using standard plate count method against the most common infection pathogens like *Staphylococcus aureus* (*S. aureus*) and *Salmonella typhi*, *Pseudomonas aeruginosa* (*P. aeruginosa*) (*P. aeruginosa*). The resulting ZnO /MWCNTs nanocomposite were characterized by analyzing the scanned electron microscopy images (SEM, the VEGA EasyProbe) and X-ray diffraction pattern (XRD, 6000-Shimadzu; Cu(Kα) spectra, λ = l.54 Å) was used to determine the crystalline structure and average size of ZnO nanoparticles and the composition of the ZnO/MWCNT nanocomposite powders.

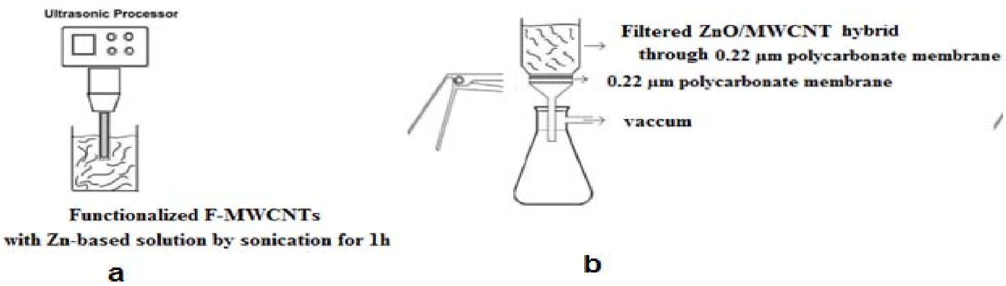
Experimental details

Preparation of ZnO/MWCNT hybrid

First step is the MWCNTs (0.1g) powder (with purity of 95%) were chemically functionalized by sonicating in a mixture of nitric acid (HNO_3) and sulphuric acid (H_2SO_4) at a 1:3 ratio (100ml) for 30 minutes to incorporate –COOH and –OH groups onto the MWCNT. The mixture was then diluted with distilled water and then filtered using through a 0.22 µm polycarbonate membrane. The functionalized MWNTs were dried under vacuum at temperature 90^0C.

Second step is preparation of the Zn-based solution by dissolving zinc acetate ($Zn(CH_3COO)_2.2H_2O$) in ethanol (C_2H_5OH) with 0.3 M ratio. Certain amount of monoethanolamine (MEA) was added to solutions to change acid-base media and all chemicals were stirred for 1 h to form a clear, stable and homogeneous sol at 60 ^{o}C. Then, the functionalized F-MWCNTs were dispersed Zn-based solution by sonication for 1h.

Then, the mixture of ZnO/MWCNT solution was vacuum-filtered through a 0.22 µm polycarbonate membrane having controlled porosity. Vacuum pressure was kept under control during production. Fig (1) shows the method of filtration ZnO/MWCNT hybrid. Finally, the ZnO/MWCNT nanocomposite was dried at 100 ^{o}C for 10 min and peeled-off from the filtration membrane. The free-standing ZnO/MWCNT nanocomposite was post-heated at 400 ^{o}C for 1 h.



Figure(1): Schematic diagram of method for preparing ZnO/MWCNTs hybrid .

Characterization of (ZnO/MWCNTs) hybrid

The phase analysis of the samples was examined by X-Ray Diffractometer (XRD, 6000-Shimadzu) equipped with Cu-Kα and radiation wavelength λ=0.15418 nm. Scanning Electron Microscopy (SEM) micrographs of the composites were also studied using (SEM, the VEGA EasyProbe), with resolution of 3 nm at 30kV and accelerating voltage 200 V to 30kV. Besides, Scanning electron microscopy (SEM, the VEGA EasyProbe) was introduced to observe the morphology of bacteria. Samples were fixed with isotonic saline and prepared by putting a 10 μl droplet on a glass chip and drying. The cells were coated with silver paste for SEM imaging.

Evaluation of antibacterial activity of (ZnO/MWCNTs) hybrid

The bacterial species used here included Gram negative Salmonella typhi (S. typhi), Pseudomonas aeruginosa (P. aeruginosa) and Gram positive Staphylococcus aureus (S.aureus). Bacteria were maintained on nutrient agar at 37^{o}C. Gram negative and Gram positive strains were provided by bio-nanotechnology lab/center of nanotechnology and advanced materials/university of Technology/Iraq.

The bacterial suspensions were prepared by taking a single colony from each stock bacterial culture with a loop and impregnated in sterile 10 ml of 0.9% normal saline. Then, the bacterial suspensions were diluted using a portion of 0.9% normal saline to obtain cell samples with concentration ~10^{7}-10^{8} CFU/ml by 0.5 McFarland standards.

The antibacterial test

For the antibacterial test, the reduction in viable cell number after interaction was determined by the **standard agar dilution method** as follows:

1 ml of bacterial solution was add to desired ZnO/MWCNT hybrid solutions at the concentrations of (0.125, 0.5, 2, 4) mg/ml. The equal mixture of saline and bacteria was as control. The mixtures were cultivated at 37 ^{0}C and shacked at 160 rpm for designed treatment times.

The suspension was serially diluted serially (1:10) with normal saline (100μl spread out on a solid nutrient agar medium) using spread plate technique. Colonies were counted after 24 h incubation of the plates at 37 ^{0}C.

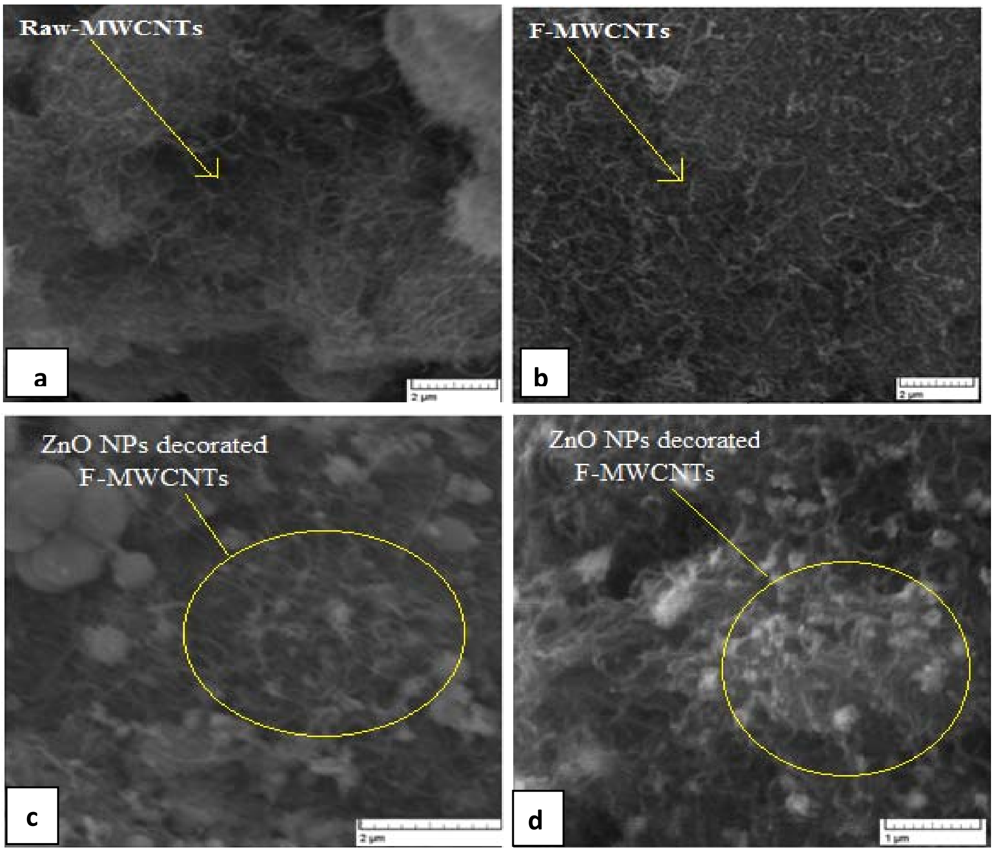
The formula to calculate the bacteriostatic rate is as following:

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

The Morphology and crystallographic structure of MWCNTs and (ZnO/MWCNTs) hybrid

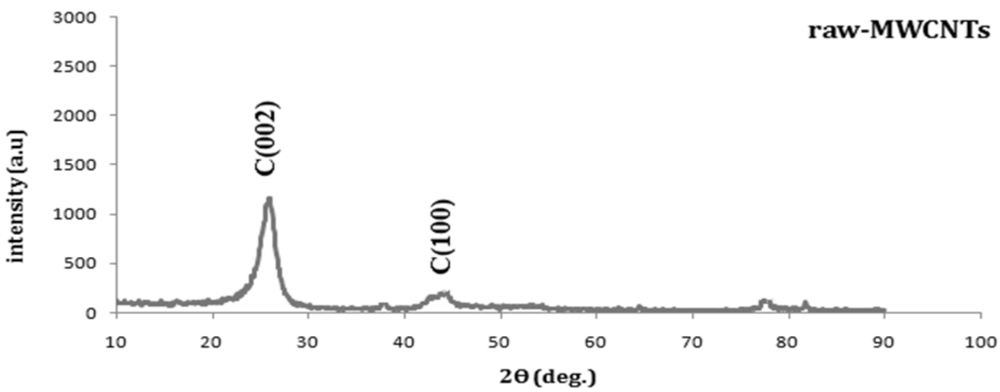
SEM micrograph at different magnifications in Fig (2 a,b),shows the morphology of raw and functionalized-MWCNTs before and after oxidation, respectively . The nanotubes are pure and only carbon nanotubes were observed with average diameter 63 nm. After treatment with acids, a change in diameter and surface roughness along the tube walls was observed. Through the oxidation process, the diameters of MWCNTs were narrowed down gradually with average diameter 60-58nm.However; oxidation of carbon nanotubes was used as a common step in the functionalization process to increase their solubility and compatibility with different materials.

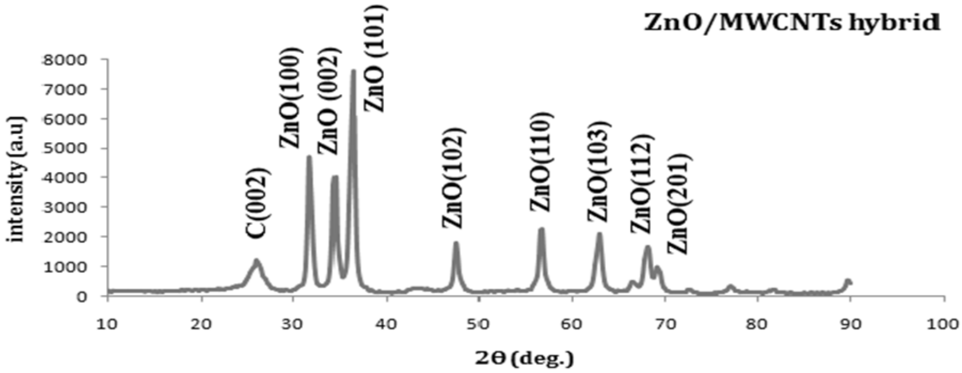
In Fig (2 c,d), shows good overall of Large Zinc oxide agglomerations were rarely observed . ZnO nanoparticles are grown in the pores and on the surface of MWCNTs, respectively. As can be seen from the higher magnification images, the MWCNT surface is covered with nanoparticles and also they agglomerate on the tubes and showing bare MWCNTs and random aggregation of ZnO onto the MWCNTs surface with particles size range (20-100nm). However, it was interesting to note that some of ZnO particles were embedded into the tube of MWCNTs in this present work.



Figure(2): SEM images of a) raw-MWCNTs, b) F-MWCNTs and c,d,) ZnO/MWCNTs hybrid at different magnifications.

The X-Ray Diffractometer (XRD) patterns of raw-MWCNTs and ZnO/MWCNTs hybrid are shown in Fig 2.The two peaks at 2Θ = 26° and 44.3° were, respectively assigned to diffraction from C (1 0 0) and C (0 0 2) planes of the MWCNTs without ZnO nanoparticles as shown in Fig 3 (a).



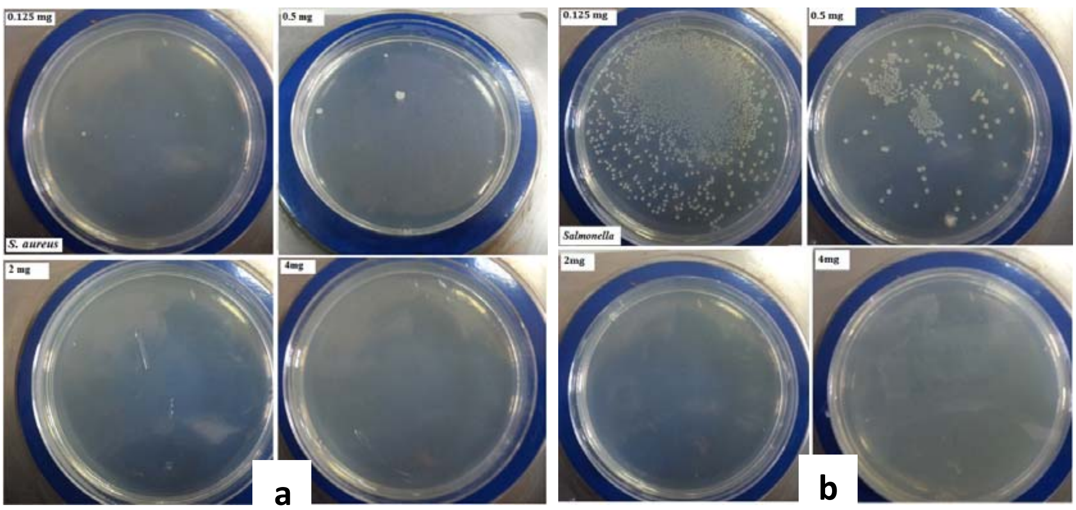


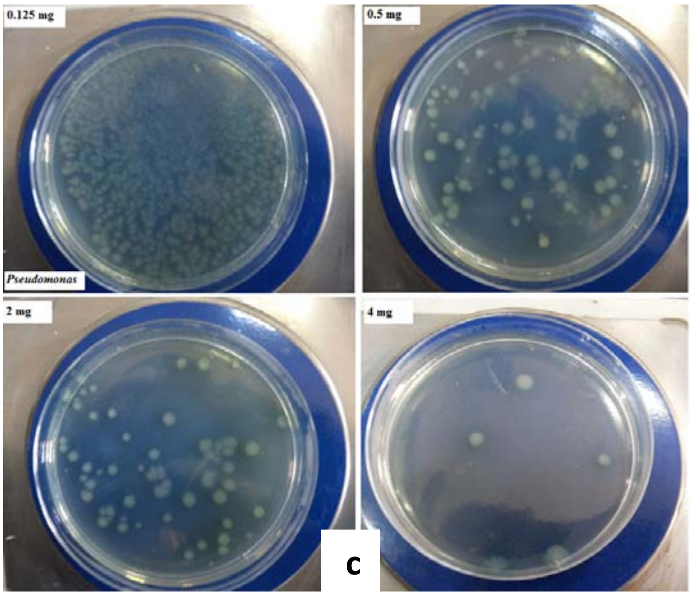
Figure(3): XRD pattern of a) row-MWCNTs, b) ZnO/MWCNTs hybrid.

The planes (1 0 0), (0 0 2), (1 0 1),(1 1 0), (1 1 2) represented the diffraction peaks of ZnO in the hybrid materials corresponding to 2Θ=31.8°, 36.3°, 34.5°, 56.6°, 68.0°, respectively besides to other diffraction peaks (1 0 2), (1 0 3), (2 0 0) and (2 0 1) of ZnO corresponding to 2Θ=47.5° ,62.9° ,66.4°,69.1° indicated crystallization of the ZnO in the hybrid and compared with the ASTM (American Society of Testing Materials) cards as shown in Fig 3 (b).

Evaluation of antibacterial activity of (ZnO/MWCNTs) hybrid

The results of antibacterial activity were shown in Fig (4). *Salmonella typhi, P.aeruginosa* and *S. aureus* served as a model of microbes for assessing the bacterial toxicity of (ZnO/MWCNTs) hybrid. The antibacterial activity of hybrid with different concentrations was estimated by counting the number of colonies growing on the plate.





Figure(4): Photograph of the colonies number (CFU) of a) S.aureus, b) Salmonella typhi and c) P.aeruginosa incubated for 24 h at 37 ^{0}C corresponding to different concentrations of ZnO/MWCNTs**: 0.125mg, 0.5mg, 2mg, 4mg**

As shown in Fig (4 a,b,c), which presents the number plate count method corresponding to increasing concentrations of (ZnO/MWCNTs) hybrid with *S. aureus, Salmonella typhi,* and *P.aeruginosa*, respectively. From the results, it's found that no bacterial colony growing on plate corresponding to high concentrations as shown with *S. aureus,* and *Salmonella typhi* in Fig (4 a,b) ,while a few bacterial colonies presented on the plates corresponding to increasing ZnO/MWCNTs hybrid as shown with *P.aeruginosa* in Fig (4 c). Since, the results shows that ZnO/MWCNTs hybrid provide a strong antibacterial property against *S. aureus* at low and high, while show strong antibacterial property at high concentrations against *Salmonella typhi,* and *P.aeruginosa*, respectively as shown in Fig (5) that represented the number of the live bacteria colonies as a function of concentrations of (ZnO/MWCNTs) hybrid with S. aureus, Salmonella typhi, and P.aeruginosa, respectively.

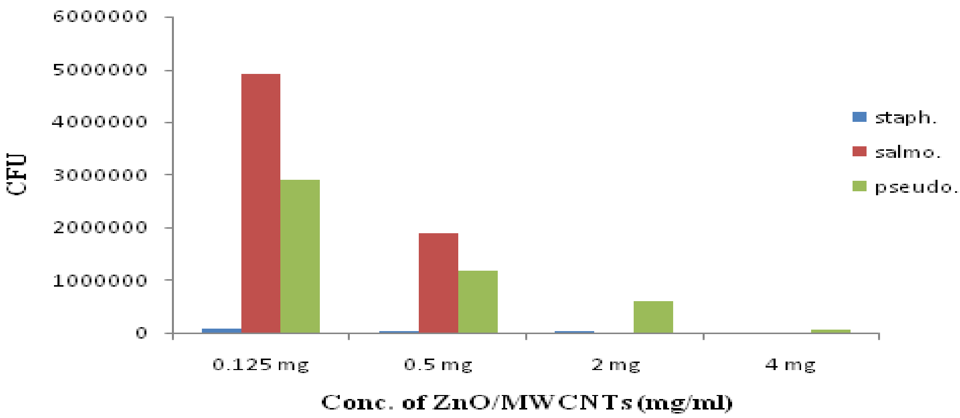


Figure (5): Number of colony counted on nutrient agar as a function of concentrations of (ZnO/MWCNTs) hybrid (CFU/ml).

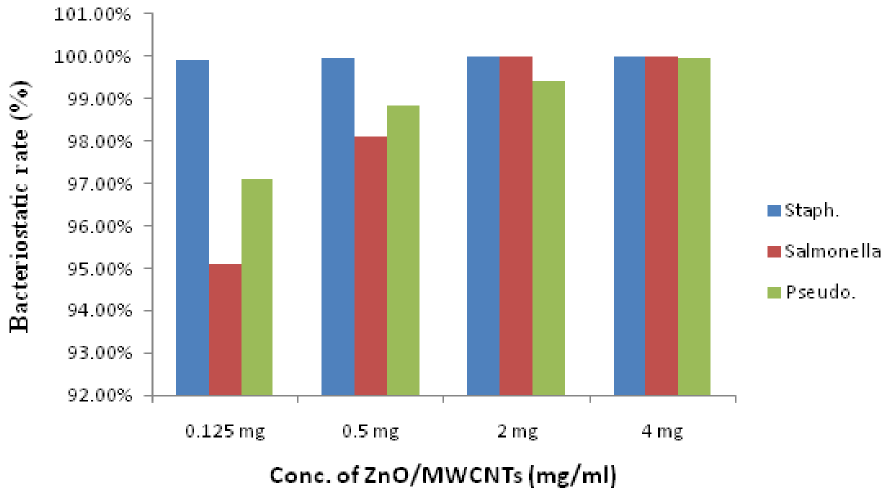


Figure (6): The bacteriostatic rate (%) of hybrid material against Salmonella typhi, P.aeruginosa and S. aureus in the 0.125mg, 0.5 mg, 2mg, 4mg of ZnO/MWCNTs concentration, respectively.

For all bacteria, the antimicrobial activity has been attributed to the damage of the cell membranes, which leads to leakage of cell contents and cell death after exposure to different concentrations of hybrid. As the results in Fig (6), more than 99% bacteria were killed after treatment with hybrid materials for 24h.

From the results in Fig (6), the bacteriostatic rate caused by hybrid material at low and high concentrations was 99.3%-100% for *S. aurous*, 95.1%-100% for *Salmonella typhi* and 97.1%-99.95% for *P. aeruginosa*. They were not much different in bacteriostatic rates at high concentration, while the bacteriostatic rate was not consistent at lower concentration. These results ravels that ZnO/MWCNTs hybrid has stronger antibacterial against *S. aureus* than *Salmonella typhi* and *P. aeruginosa*. Although the accurate mechanism of killing is still unknown, the production of strong oxidizing agent harmful to the cells of living organisms from the ZnO surface has been considered as the key factor of antibacterial activity of ZnO nanocomposites, against Gram-negative and Gram-positive bacteria. Therefore, the formation of ZnO-C bond between ZnO and MWCNTs resulting in the death of the bacteria by penetrating the cell wall and membrane and inhibiting their breeding. At same time, causing their cytoplasm to run off and oxidizing their cell nucleus. Therefore, a good interfacial combination between ZnO and MWCNTs should also promote the charge transfer between ZnO and MWCNTs to obtain a ZnO/MWCNTs nanocomposite with a synergistic property.

Scanning Electron Microscopy (SEM) images of bacteria

Fig (7) shows SEM images of each *S. aureus Salmonella typhi, and P.aeruginosa* in fundamental shape and the bacterial cells adsorption with hybrid materials at concentration 4mg/ml.

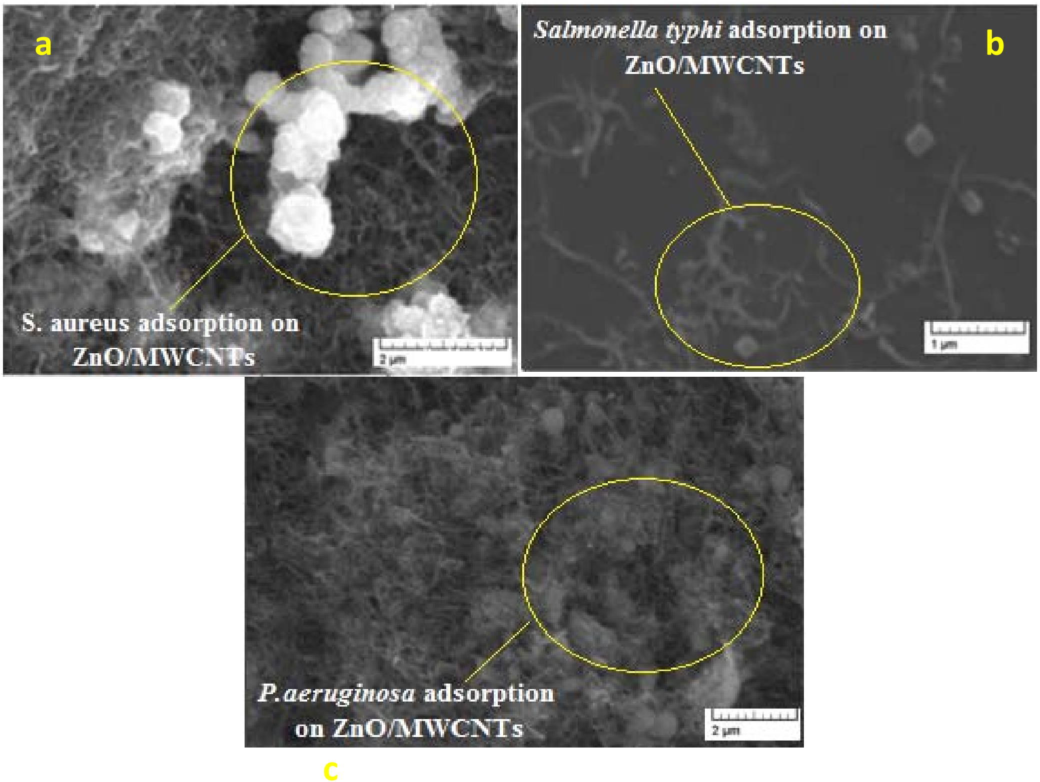


Figure (7): SEM images of a) *S. aureus*,b) *Salmonella typhi,* and c) *P.aeruginosa* on a slide glass after mixing with hybrid materials samples at a concentration of 4mg/ml

In Fig (7 a,b,c), which represented the adsorption of each *S. aureus* (a), *Salmonella typhi* (b) and *P.aeruginosa* (c) with ZnO/MWCNTs of concentration 4mg/ml and also shows the cell interaction with hybrid material .

CONCLUSION

We have demonstrated a simple and reproducible chemical method to modify MWCNTs with ZnO nanoparticles using monoethanolamine (MEA) as the solvent and stabilizer as shown in XRD and SEM analysis. ZnO/MWCNTs hybrid materials at different concentrations and their antibacterial activity against Gram-negative and Gram-positive bacteria were studied. The combination of ZnO nanoparticles and MWCNTs altered their toxicity and antibacterial properties and showed excellent bactericidal potential. Overall, results demonstrate the great potential of the developed ZnO/MWCNTs nanocomposites to prevent microbial contamination, which is of great interest for food packaging applications.

Acknowledgements

Authors gratefully acknowledge nanotechnology and advanced material research centre, University of Technology, Baghdad, Iraq for conducting the SEM (VEGA EasyProbe), X-Ray diffractometer (XRD, 6000-Shimadzu) and biotechnology lab for making different antibacterial activity tests and for their help and support.

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