Video Article

Synthesis of Multi-walled Carbon Nanotubes Modified with Silver Nanoparticles and Evaluation of Their Antibacterial Activities and Cytotoxic Properties

Youngmin Seo*[1], Chanhwi Park*[2], Jaewoo Son[2], Kyungwoo Lee[2], Jangsun Hwang[2], Yeonho Jo[2], Dohyun Lee[2], Muhammad Saad Khan[2], Sachin Ganpat Chavan[2], Yonghyun Choi[2], Dasom Kim[2], Assaf A Gilad[3], Jonghoon Choi[2]

[1]Center for Biomaterials, Biomedical Research Institute, Korea Institute of Science and Technology

[2]School of Integrative Engineering, Chung-Ang University

[3]Division of Synthetic Biology and Regenerative Medicine, Institute for Quantitative Health Science and Engineering, Michigan State University

*These authors contributed equally

Correspondence to: Assaf A Gilad at gilad@msu.edu, Jonghoon Choi at jonghoonc@gmail.com

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Abstract

In this study, multi-walled carbon nanotubes (MWCNTs) were treated with an aqueous sulfuric acid solution to form an oxygen-based functional group. Silver MWCNTs were prepared by the reductive deposition of silver from an aqueous solution of $AgNO_3$ on the oxidized MWCNTs. Given the unique color of the CNTs, it was not possible to apply them to the minimum inhibitory concentration or mitochondrial toxicity assays to evaluate the toxicity and antibacterial properties, since they would interfere with the assays. The inhibition zone and minimum bactericidal concentration for the Ag-MWCNTs were measured and Live/Dead and Trypan Blue assays were used to measure the toxicity and antibacterial properties without interfering with the color of the CNTs.

Video Link

The video component of this article can be found at https://www.jove.com/video/57384/

Introduction

The ultimate goal of this study is to make environmentally friendly antibacterial nanomaterials that can inhibit the growth of bacteria that form biofilms. These antibacterial nanomaterials have the potential to overcome the toxicity and antibiotic resistance problems of commonly used chemicals or antibiotic chemical compounds. A biofilm is a hydrated extracellular polymeric substance (EPS) that is composed of polysaccharides, proteins, nucleic acids, and lipids[1,2]. Biofilms prevent the intrusion of foreign substances and help bacteria grow vigorously[3,4]. Biofilms cause odor and chronic infectious diseases[5,6]. *Methylobacterium spp*., for example, grows by adhering to places where water is always present or where it is difficult to ensure bacterial eradication on a continual basis, such as air conditioner heat exchangers, shower rooms, and medical devices. These types of biofilms cause odor and chronic infectious diseases[5,6].

Typically, chemicals or antibiotic chemical compounds are used to inhibit the growth of bacteria that form biofilms. The emergence of antibiotic resistant bacteria and concerns about *in vivo* safety of chemicals are driving the need to develop new materials to prevent the formation of biofilms and to inhibit the growth of bacteria.

In this study, antimicrobial nanomaterials are synthesized that are free from antibiotic resistance and toxicity. Silver is a well-known antimicrobial substance, and recent developments in nanoscience and nanotechnology have led to active research into the antimicrobial effects of metal nanoparticles[7,8]. Recent studies have reported that the small size and high surface-to-volume ratio of the nanoparticles result in increased antibacterial activity[9,10,11].

The nanomaterials presented herein combine silver nanoparticles with increased antimicrobial properties and carbon nanotubes with a high aspect ratio, thereby increasing the surface area per unit volume. The fabricated silver nanoparticle-carbon nanotube composite exhibits substantial antimicrobial properties and minimal toxicity to human and animal cells. The synthetic processes in previous studies use hazardous reducing agents such as $NaBH_4$, formamide, dimethylformamide, and hydrazine. The process is complicated, dangerous, and time-consuming. The synthetic process reported here uses ethanol as a significantly less hazardous reducing agent.

The inhibition zone and minimum bactericidal concentration (MBC) for the Ag-MWCNTs were measured; Live/Dead and Trypan Blue assays were used to measure toxicity and antibacterial properties. Minimum inhibitory concentration (MIC) and mitochondrial toxicity (MTT) assays were

not performed due to the unusual color of the carbon nanotubes which would have interfered with the assays. Finally, the minimum concentration to prevent the growth of *Methylobacterium spp.* without affecting mammalian cells was determined.

Protocol

1. MWCNT Oxidation

1. Measure 30 - 50 mg of MWCNT into a 50-mL vial. Slowly add 8 mL of a H_2SO_4:HNO_3 solution (90% initial concentration, 3:1 vol/vol) by pipette with 1 mL pipette tips.
   CAUTION: This preparation must be conducted in a chemical fume hood.
   1. Allow 30 min for the exothermic reaction to complete.
2. Sonicate the solution at 60 - 80 °C and 160 W for 1 h until MWCNT settles at the bottom of the vial.
   CAUTION: The water level in the sonicator should be lower than the top of vial so that water will not be introduced into the vial.
3. Transfer the solution to a centrifuge tube.
   1. With a micropipette, add 30 mL of distilled water dropwise to the MWCNT-COOH solution.
      CAUTION: A strong exothermic reaction occurs during addition of distilled water. Add distilled water slowly and allow time for cooling.
4. Centrifuge the MWCNT-COOH solution at 12,000 x g and room temperature for 15 min.
   CAUTION: When operating a centrifuge, ensure the centrifuge remains balanced by placing a tube with the same weight opposite the sample tube.
5. Remove the supernatant with a pipette and 1 mL pipette tip. Add 5 mL of ethanol with a pipette and 1 mL pipette tip. Rinse the walls of the vial with additional pipetted ethanol. Centrifuge the solution at 12,000 x g and room temperature for 15 min.
   1. Determine pH of supernatant with pH paper. Repeat washing and centrifuging until the supernatant pH is neutral.
6. Remove all supernatant. Add 1 mL of distilled water to precipitate and disperse MWCNT-COOH evenly in solution. Freeze-dry solution at -60 °C under vacuum until dry.

2. Silver Nanoparticle Deposition on MWCNTs

1. Place 10 mg of MWCNT-COOH in a 50-mL conical tube and dilute with 30 mL of distilled water. Sonicate solution at 60 °C and 160 W for 1 h.
2. Place 6 mL of 0.1 N $AgNO_3$ in a 50-mL conical tube and dilute with 18 mL of distilled water. Sonicate solution at 60 °C and 160 W for 1 h.
   CAUTION: Add $AgNO_3$ in proportion to the total mass of MWCNTs such that the total mass of Ag does not exceed 20%.
3. Add the $AgNO_3$ solution dropwise to the MWCNTs solution with a pipette and 1 mL pipette tip while sonicating the mixture at 60 °C and 160 W. Continue sonicating for 1 h after adding the $AgNO_3$.
4. Centrifuge the solution at 12,000 x g and room temperature for 15 min and remove the supernatant. Centrifuge the solution and remove the supernatant two additional times.
5. Add 1 mL of distilled water to the Ag-MWCNTs mixture and disperse the precipitate evenly in the solution. Add 5 mL of ethanol and allow the reaction to proceed at room temperature for 1 h.
6. Centrifuge the solution at 12,000 x g and room temperature for 15 min and remove the supernatant.
7. Determine pH of supernatant with pH paper. Repeat washing and centrifuging until supernatant pH is neutral.
8. Add 1 mL of distilled water to Ag-MWCNTs and disperse evenly in solution. Freeze-dry the solution at -70 °C under vacuum for 24 h.

3. Zone of Inhibition Test

1. Mix 18.12 g of R2A agar and 1 L of distilled water in a flask. Sterilize the mixture in an autoclave at 121 °C for 15 min.
2. Allow gel to cool at room temperature. Place 10 mL of gel in Petri dishes prior to hardening to prepare the solid medium.
3. Place 100 µL of bacteria on the solid medium. Spread the bacteria on the solid medium using a spreader.
   CAUTION: This procedure must be conducted in a chemical fume hood lit with an alcohol lamp.
4. Incubate the dishes at 30 °C for 48 h.
5. Mix 3.12 g of R2A broth with 1 L of distilled water in a flask. Sterilize the mixture in an autoclave at 121 °C for 15 min.
6. Transfer the grown colonies to a liquid medium using a loop and needle and incubate in an incubator at 180 rpm and 30 °C.
7. Record the OD (Optical Density) value by UV spectrophotometry at 600 nm on an hourly basis to measure bacterial growth.
   NOTE: R2A agar was used in this test, rather than the standard Mueller-Hinton agar, because R2A is the preferred agar for growth of *Methylobacterium spp.*
8. Place 10 mL of prepared R2A agar in a Petri dish to prepare a bottom agar.
   1. Mix 3.12 g of R2A broth and 8 g of agar with 1 L of distilled water. Sterilize the mixture in an autoclave at 121 °C for 15 min. Place 10 mL of this gel on the bottom agar.
9. Load 50 µL of Ag-MWCNTs solution on sterile paper disc with a micropipette.
   1. Dry and sterilize Ag-MWCNTs sample in a vacuum chamber under UV light for 30 min.
10. Place Ag-MWCNTs sample on agar.
11. Incubate the agar plate at 30 °C for 48 h.
12. Measure zone of inhibition[12].
    NOTE: Instructions for software used are included in the references.

4. Antibacterial Test

1. Repeat steps 3.1 to 3.7 to prepare bacterial culture.
2. At maximum OD, inoculate 100 µL of bacteria into 3 mL of fresh liquid medium with a pipette and 100-µL micro pipette tip.
3. Prepare five 50 µL samples of the control solution in 15-mL conical tubes. Add the following to each tube: 1) methanol; 2) 1000 µg/mL MWCNT-COOH; 3) 50 µg/mL Ag-MWCNTs; 4) 40 µg/mL Ag-MWMWCNTs; 5) 30 µg/mL Ag-MWCNTs.
4. Incubate at 180 rpm and 30 °C until the control is maximally active as determined by UV spectrophotometry as described above.
5. Measure the OD value of each sample and calculate the MIC concentration. The OD value is directly related to the number of bacterial cells in the sample broth.
6. Spread the cultured bacteria on a solid medium with a spreader.
7. Incubate the dish at 30 °C for 48 h.
8. Count bacterial colonies and the measure CFU values to determine antibacterial activity[12].
   NOTE: Instructions for software used are included in the references.

5. Viability Assay

1. After removing the culture cells from incubator, suction the medium and wash three times with a dropwise wash of 5 mL of PBS.
2. Add 1 mL of trypsin-EDTA in PBS to the washed cell and suction after 1 min.
   1. Tap the plate to remove stuck cells.
3. Add 5 mL of DMEM (Dulbecco's Modified Eagle's Medium) and then remove the cells. Centrifuge the samples at 200 x g for 3 min and remove the supernatant.
   1. Add 1 mL of DPBS and mix.
   2. Count cells in 10 µL of the solution with an automated cell-counting machine.
      NOTE: Instructions for cell-counting machine used are included in the references[13].
4. Place 1 × 10^5 of AML12 cells per well in a six-well plate containing DMEM solution. The medium contains 10% (v/v) fetal bovine serum and 1% sterile antibiotic.
5. Incubate plate for 8 - 12 h at 37 °C in a 5% CO_2/95% air environment.
6. Suction the supernatant from wells with an aspirator.
   1. Add each sample comprised of control, NMS-DMSO (dimethyl sulfoxide) and 30-40 µg/mL Ag-MWCNT to 1 mL of DMEM.
   2. Incubate at 37 °C in a 5% CO_2/95% air environment for 8 h.
7. Remove supernatant and wash samples with 5 mL of PBS.
8. Add 1 mL of prepared live/dead kit (20 µL of 2 mM EthD-1 with 5 µL of 4 mM calcein AM in DMSO in 10 mL of PBS) dropwise to the cell.
9. Incubate at room temperature for 30 - 45 min or 37 °C for 10 min. Measure fluorescence with standard fluorescence microscopy at 100X or 300X.

6. Trypan Blue Assay

1. Prepare samples according to steps 5.1 through 5.6.2 above.
2. Remove supernatant.
3. Wash plate with 1 mL of 1x DPBS and decant.
4. Add 1 mL of trypsin to plate and incubate for 3 min to detach the cells from culture. Use cell culture medium to wash plate and collect cells. Place collected cells in a 15-mL tube.
5. Centrifuge tube at 200 x g and 4 °C for 3 min.
6. Discard supernatant. Add 1 mL of fresh medium to cell pellet and re-disperse the cells.
7. Add 10 µL of trypan blue with 10 µL of dispersed cells and count the cells with an automated cell-counting machine.
   NOTE: Instructions for cell-counting machine used are included in the references.

Representative Results

Transmission Electron Microscopy (TEM) images confirm the formation of Ag-MWCNTs (**Figure 1A and 1B**). Their successful synthesis was confirmed by the change in surface charge. The size of the Ag particles deposited on the MWCNTs was calculated (**Figure 1C**). The average particle size was approximately 3.83 nm. The XRD pattern of the as-synthesized Ag-MWCNTs is shown in **Figure 1D**. The peak at 20 - 30° corresponds to MWCNT; the remaining peaks correspond to Ag. Antimicrobial activity data is shown in **Figure 2**. Bacterial colony populations were confirmed by *Methylobacterium* control (**Figure 2A**); addition of methanol reduced the population 10^3 times (**Figure 2B**), and addition of MWCNT-COOH reduced the population 10^8 times (**Figure 2C**). Colonies could not be identified in the 50 µg/mL Ag-MWCNT (**Figure 2D**) and 40 µg/mL Ag-MWCNT (**Figure 2E**) samples. In the 20 µg/mL Ag-MWCNT sample the population was reduced 10^5 times (**Figure 2F**). In the zone of inhibition test against *Methylobacterium* (**Figure 3**), the zone of inhibition was identified when 10 µg/mL was added. A zone of inhibition was not observed at a concentration of 1 µg/mL. In the cytotoxicity test, the Live/Dead assay (**Figure 4**) confirmed the absence of cytotoxicity in the negative control (**Figure 4A**) and the presence of cytotoxicity in the positive control (**Figure 4B**) when methanol was used. The addition of 40 µg/mL Ag-MWCNTs (**Figure 4C**) revealed some cytotoxicity, however the addition of 30 µg/mL Ag-MWCNTs (F**igure 4D**) did not reveal a significant amount of cytotoxicity. A trypan blue assay was performed (**Figure5**) for the control (**Figure 5A**), methanol (**Figure 5B**), 40 µg/mL Ag-MWCNTs (**Figure 5C**) and 30 µg/mL Ag-MWCNTs (**Figure 5D**) samples. The results confirmed that there was very little cytotoxicity at 30 µg/mL Ag-MWCNTs.

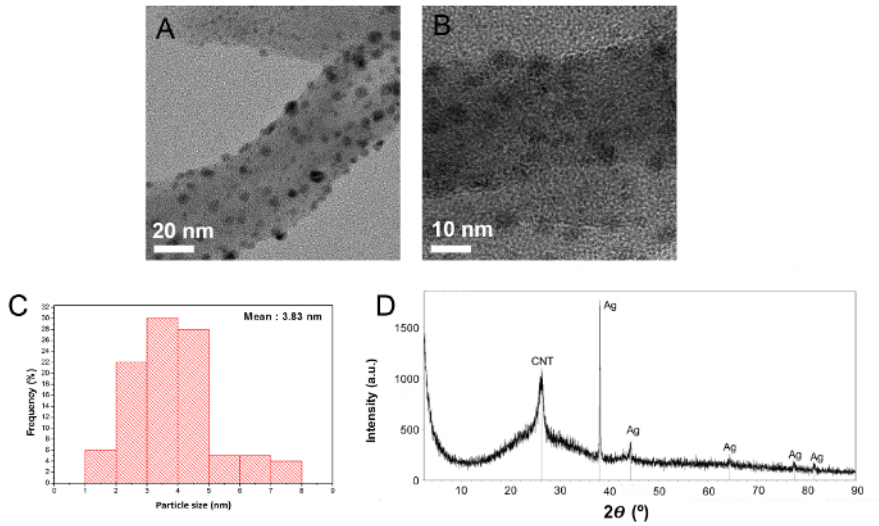

Figure 1: Physicochemical characterization of Ag-MWCNTs. (A) and (B) TEM images of Ag-MWCNTs; **(C)** size distribution of Ag-MWCNTs as assessed via TEM (n = 100); **(D)** XRD pattern of Ag-MWCNTs. Please click here to view a larger version of this figure.

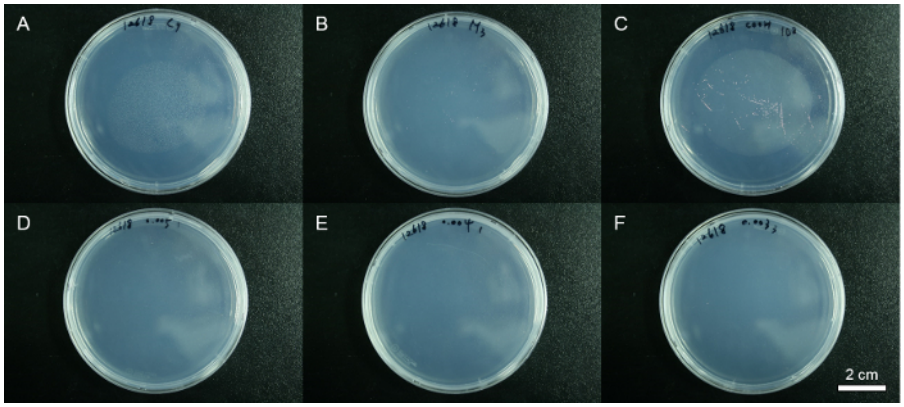

Figure 2: Antibacterial evaluation of Ag-MWCNTs relative to *Methylobacterium spp.* (A) control, **(B)** methanol, **(C)** MWCNT-COOH, **(D)** 50 µg/mL of Ag-MWCNTs, **(E)** 40 µg/mL of Ag-MWCNTs, and **(F)** 30 µg/mL of Ag-MWCNTs. Please click here to view a larger version of this figure.

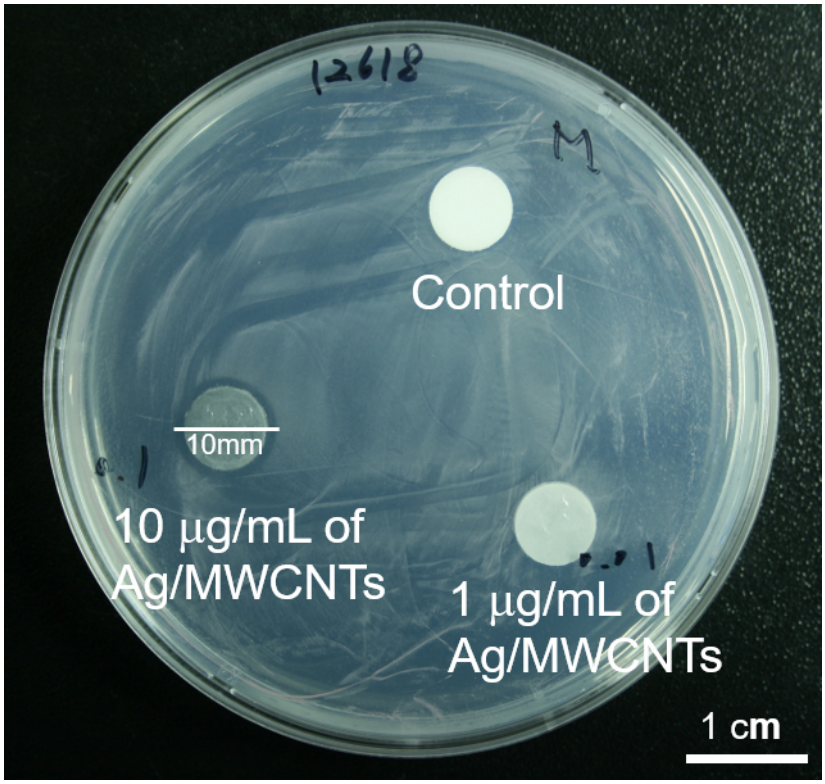

Figure 3: Zone of inhibition test of Ag-MWCNTs.

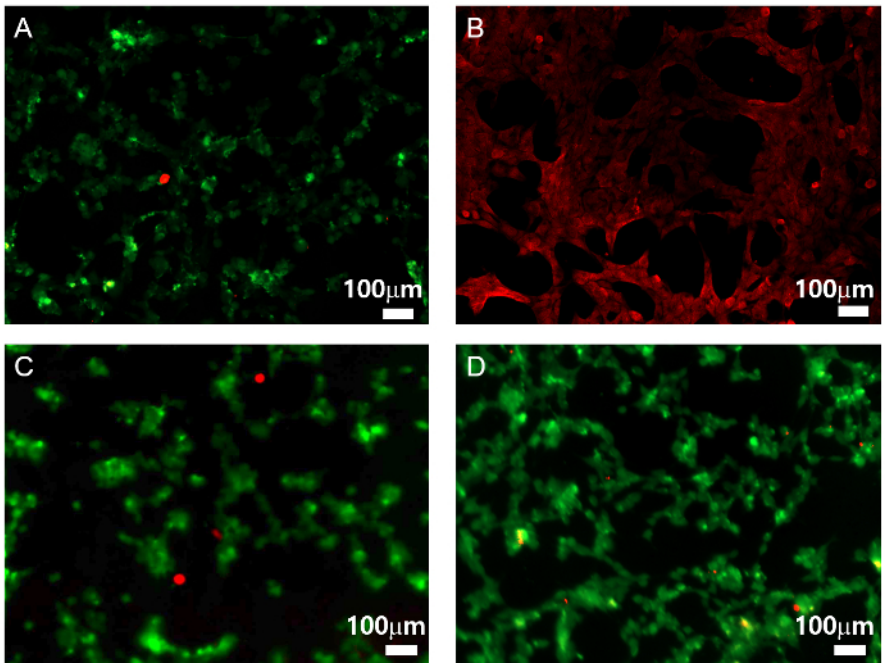

Figure 4: Cell viability assays for Ag-MWCNTs. Photomicrographs of AML12 that are fluorescently labeled for dead (red) and live (green) cells. (A) control, **(B)** methanol, **(C)** 40 µg/mL of Ag-MWCNTs, and **(D)** 30 µg/mL of Ag-MWCNTs. Please click here to view a larger version of this figure.

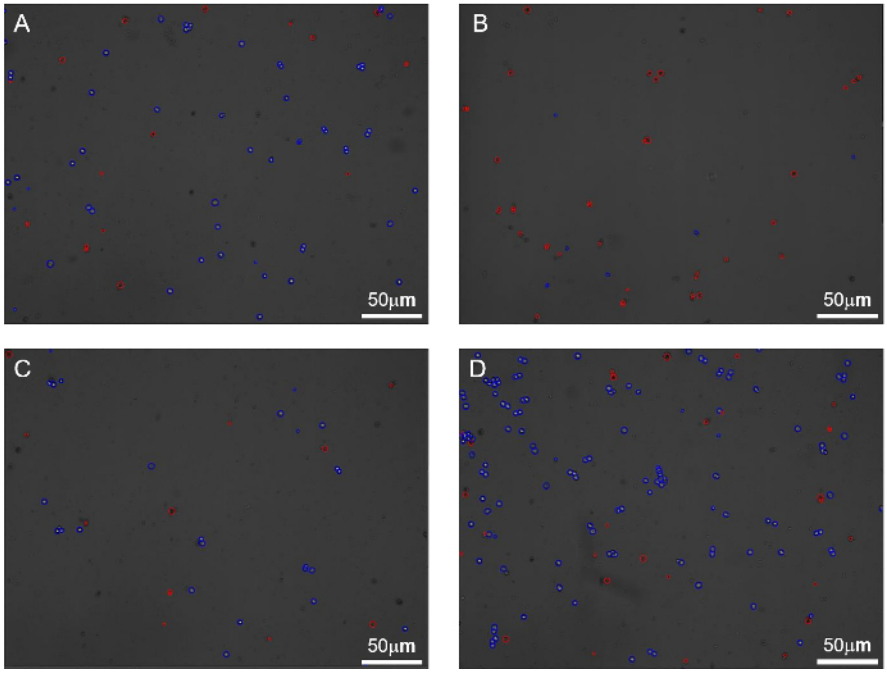

Figure 5: Trypan blue assays for 30 and 40μg/mL Ag-MWCNTs. (A) control, **(B)** methanol, **(C)** 40 μg/mL of Ag-MWCNTs, and **(D**) 30 μg/mL of Ag-MWCNTs. (Red: dead cells; blue: live cells) Please click here to view a larger version of this figure.

Discussion

Here, we report a simple method for the preparation of MWCNTs with deposited Ag nanoparticles. This silver-containing nanomaterial demonstrates substantial antibacterial activity and minimal potential for uncontrolled absorption of silver nanoparticles in the body. We demonstrate that 30 μg/mL of synthesized Ag-MWCNTs is an effective level of antibacterial activity against *Methylobacterium spp.* with negligible cytotoxicity to mammalian liver cells. Though additional improvements and biosafety assessments for Ag-MWCNTs are required before expansion in the commercial sector, using ethanol as a reducing agent could help produce environmentally-friendly and inexpensive Ag-MWCNTs.

The following points are demonstrated. It is possible to destroy various types of bacteria with the developed nanomaterials. It is possible to engineer multiple sites on a nanotube surface upon which silver nanoparticles can be deposited. Antibacterial properties can also be tailored by controlling the size and number of silver nanoparticles that are deposited on the surface. The developed nanomaterials are toxic to bacterial cells but are nontoxic to human and animal cells in appropriate concentrations.

The following mechanism is proposed for the antibacterial activity. The Ag-MWCNT nanostructures directly contact the bacterial cell surface, damage the cell wall, and cause secondary oxidation of reactive oxygen species; these processes result in oxidative stress. Ag-MWCNT nanostructures have been confirmed to release silver ions that inhibit the quorum sensing of *Methylobacterium* spp., thereby inhibiting the expression of the genes that govern the formation of biofilms.

The limitations of the current protocol would include the undetermined maximum amount of silver nanoparticles and Ag-MWCNTs allowable in human trials. In this protocol, the amount of silver nanoparticles included in the 30 μg/mL sample of Ag-MWCNTs was estimated as 0.4 μg/mL, on average. This concentration is regarded as biocompatible with the mammalian cells as reported in previous studies[6,7,8,9]. While the mechanism for cytotoxicity at the concentration of 40 μg/mL of Ag-MWCNTs is undetermined, it is proposed that the non-attachment of blood cells to the bottom of culture plates may increase the likelihood of Ag-MWCNT contact during culture. In the future, detailed toxicity studies of silver nanoparticles with a variety of cell types under different culture conditions can be performed. These studies may provide additional information on the mechanism of Ag-MWCNT interaction with cells.

Disclosures

The authors have nothing to disclose.

Acknowledgements

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References

1. Löndahl, J. Physical and Biological Properties of Bioaerosols, in *Bioaerosol Detection Technologies.* Springer. 33-48 (2014).
2. Jennings, S., A. Moran, and C. Carroll, Bioaerosols and biofilms. *Biofilms in medicine, industry and environmental biotechnology.* IWA, London, 160-178 (2003).
3. Flemming, H.-C. and J. Wingender, The biofilm matrix. *Nature Reviews Microbiology.* **8** (9), 623-633 (2010).
4. Lewis, K., Riddle of biofilm resistance. *Antimicrobial agents and chemotherapy.* **45** (4), 999-1007 (2001).
5. Doronina, N.V., *et al.* Methylobacterium suomiense sp. nov. and Methylobacterium lusitanum sp. nov., aerobic, pink-pigmented, facultatively methylotrophic bacteria. *International Journal of Systematic and Evolutionary Microbiology.* **52** (3), 773-776 (2002).
6. Seo, Y., *et al.* Antibacterial activity and cytotoxicity of multiwalled carbon nanotubes decorated with silver nanoparticles. *International Journal of Nanomedicine.* **9**, 4621-4629 (2014).
7. Chen, X. and H. Schluesener, Nanosilver: a nanoproduct in medical application. *Toxicologyletters.* **176** (1), 1-12 (2008).
8. Singh, M., *et al.* Nanotechnology in medicine and antibacterial effect of silver nanoparticles. *Digest Journal of Nanomaterials and Biostructures.* **3** (3), 115-122 (2008).
9. Morones, J.R., *et al.* The bactericidal effect of silver nanoparticles. *Nanotechnology.* **16** (10), 2346 (2005).
10. Martinez-Castanon, G., *et al.* Synthesis and antibacterial activity of silver nanoparticles with different sizes. *Journal of Nanoparticle Research.* **10** (8), 1343-1348 (2008).
11. Lok, C.-N., *et al.* Silver nanoparticles: partial oxidation and antibacterial activities. *JBIC Journal of Biological Inorganic Chemistry.* **12** (4), 527-534 (2007).
12. Ferreira, T., Rasband, W. *ImageJ User Guide Home Page.* <http://imageJ.nih.gov/ij/docs/guide> (2010).
13. NanoEnTek Inc. *Juli Br User Guide.*; Hwaseong, Korea <http://www.nanoentek.com/upload/product/11/JuLI%20Br_User%20manual%20(V.1.6).pdf> (2012).

