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Research Article

Fabrication and Characterization of Multi Walled Carbon Nanotube Cross Linked with Silver Nanoparticles for Antimicrobial Activities

¹Sarmistha Swain and *Smaranika Das¹Department of Chemistry, DGBK College, Odisha, India

*Department of Botany, Revenshaw Junior College, Odisha, India

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Abstract: In present study functionalized Multi Walled Carbon Nanotube (F-MWCNT) cross linked with Silver nanoparticles using plant leaf extract *Azadirachta indica* (A-AgNPs) known as (F-MWCNT-AgNPs). The prepared nanocomposites were characterized by UV-Visible spectrophotometer, XRD, FTIR, TEM, and evaluated for their prospective antibacterial activities. The effect of F-MWCNT concentration on the A-AgNPs was studied along with their post-synthetic stability against varying pH value and long term storage. The antibacterial activities of A-AgNPs and F-MWCNT-AgNPs were studied with respect to Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli*. The growth curves, formation of bactericidal reactive oxygen species (ROS), protein leakage, and lactate dehydrogenase activity involved in the respiratory chain. These results suggest that F-MWCNT-A-AgNPs could be used as an effective antibacterial material. The current work proves the potential of nanobiotechnology that involves a fusion of advanced techniques from nanoscience to develop the biology and used in the fields of drug delivery, Bio Sensor Manufacturing, Medical therapy, etc.

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*Corresponding Author:

E-mail Address: smaranikadas74 @gmail.com (S. Das).

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1. Introduction

The infectious diseases caused by different pathogenic bacteria and the development of antibiotic resistance the pharmaceutical companies and the researchers are searching for new antibacterial agents (Engering et al., 2013). In the present scenario, nanoscale materials have emerged up as novel antimicrobial agent owing to their high surface area to volume ratio and the unique chemical and physical properties (Beyth et al., 2015).

Nanosized metal particles show notable physical, chemical and biological properties compared with bulk solids (Zhang et al., 2016). Silver nanoparticles (AgNPs) have attracted the interest of the scientific community because silver has been used as an antiseptic and antimicrobial against different species of bacteria (Jun et al., 2007). The growing resistance of pathogenic bacterial and fungal strains to traditional treatments is another key factor driving research with this type of material. In recent years, the effectiveness of some nanoconjugates has been demonstrated against some pathogenic microbes, showing great potential for nanomedicine. The combination of antibiotics, antifungal agents such as azoles, metal complexes and nanoparticles could increase the efficiency of drugs, even against drug-resistant pathogens (Kim et al., 2012). The use of the antibiotic capacity of silver has also been expanded to the field of biotechnology and bioscience for research in various fields, such as osteoimplantation, inflammation, burns treatment, drug delivery, and biochips. In particular, an enhanced level of antibacterial properties observed in nanoscale silver materials has led to increased interest in the application of silver nanomaterials in biomedical research (Adeyemi et al., 2015; Benyettou et al., 2015). The antibacterial activity of silver

nanoparticles has led to their promotion for use in medical devices.

In order to resolve this issue, we hypothesized that the integration of AgNPs into F-MWCNTs would provide maximal antibacterial properties and minimal bio toxicity. Since F-MWCNTs have a high aspect ratio, given their nanoscale diameter and microscale length, their large surface area is adequate for the deposition of mass quantities of molecules during water filtration/disinfection (Kang et al., 2008; Jha et al., 2017; Baghayeri et al., 2016; Chen et al., 2016).

In this study, we combined A-AgNPs with F-MWCNTs and expected to see a synergistic increase in antibacterial activity against Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli*. By loading A-AgNPs onto F-MWCNTs, we anticipated that the resulting F-MWCNTs-A-AgNPs could be optimized for minimal absorption into the body or excretion into the environment.

2. Material and Method:

2.1. Chemicals and Plant Material

Silver nitrate (AgNO_3) and MWCNT was obtained from Sigma-Aldrich. Ampicillin and all other chemicals were purchased from Himedia and Sigma-Aldrich. Millipore Water was used in the entire experimental work. The plant *Azadirachta indica* was obtained from Ravenshaw University (Botanical garden) Cuttack, Odisha, India.

2.2. Preparation of Leaf Extract

Fresh 100 gm *Azadirachta indica* leaves were collected and washed with triple distilled water and cleaned. Leaves were dried with the help of water absorbent paper (wet filter paper). The aqueous extracts of the leaf material were obtained in the laboratory using the Mortar and pestle. The final leaf extracted material was repeated each time a new solvent with the next higher polarity was used

to obtain the extract. The *Azadirachta indica* leaf extract is known as AIE.

2.3. Preparation of silver nanoparticles (A-AgNPs)

The AIE extract collected were stirred at a regulated speed on the magnetic stirrer. The AIE extract used here was of 100ml which was collected in a 500ml flask and the speed used was 200rpm followed by Ahmed et al., 2016. To the above solution, 10 ml of 5mM AgNO₃ was added in a slow and steady manner drop by drop. The extracts and reagents were used in the different concentration

(1:1,1:2,1:3,1:4,1:5,1:6,1:7,1:8,1:9,1:10). The stirring was continued until a change in the colour appears, and the solutions turn brownish. The prepared silver nanoparticle is known as (A-AgNPs).

2.4. Fabrication of F-MWCNT-A-AgNPs Nanocomposite

100 mg F-MWCNT was dispersed in 80 ml water by 60 min of sonication. The reaction mixture was prepared in a 250 mL round-bottom flask by dissolving 20ml of aqueous A-AgNPs with 60 ml of the F-MWCNT; the mixture was stirred at 60°C for 24h. The solutions were then mixed and sonicated for 1 hour to decorate the A-AgNPs onto the F-MWCNTs. The resultant mixture was washed three times with water using centrifugation. The product was obtained as a pure and used for further experiments (Baghayeri et al., 2016).

3. Characterization of NPs

3.1. UV-visible spectral analysis

The UV-visible spectrum from 200 to 900 nm of AIE, A-AgNPs, F-MWCNT-A-AgNPs with agar medium were recorded in an EPOCH™ Multi-Volume Spectrophotometer System (Biotek Instruments, Inc., Mumbai, India). A-AgNPs

displayed maximum absorbance at 445 nm with predomination of smaller size particles.

3.2. Transmission electron microscopy (TEM)

Morphological characterization of A-AgNPs was carried out by TEM (JEOL-JEM 2100, 1.4 Angstrom Unit, Tokyo, Japan). The A-AgNP was made by air-drying drops of diluted A-AgNP solutions on carbon films supported by copper grids. TEM images were visualized at 120 kV under the microscope.

3.3. Particle size analysis measurements

Dynamic light scattering (DLS) was used to estimate the hydrodynamic diameter, and laser Doppler anemometry was used to determine zeta-potential (mV). To define the particle size and zeta-potential, a dilute suspension of A-AgNPs (100 µl diluted to 1 ml Milli-Q water) was sonicated in an ice bath for 30s and subjected to particle size and zeta potential determination.

3.4. Fourier transforms infrared spectral studies (FTIR)

To examine the chemical functional group interactions between the *Azadirachta indica* leaf extract and AgNO₃, F-MWCNT-A-AgNPs, FTIR spectra study was taken out using a PerkinElmer Model Spectrum 1 (PerkinElmer, MA, USA). The prepared samples were mixed and crushed with potassium bromide (Sigma-Aldrich) by applying a pressure of 300 kg/cm² to prepare the pellets for analysis. An FTIR spectrum was examined in the range between 500 and 4000 cm⁻¹.

3.5. X-ray diffraction analysis (XRD)

The lyophilized powders of the A-AgNPs were used for XRD study (powder X-ray diffractometer, Bruker, USA). The diffracted intensities were reported from 0 to 80° at 2θ angles. The diffraction pattern corresponds to AgNO₃, F-MWCNT-A-AgNPs.

3.6. Determining the growth curves of bacterial cells

To examine the growth curves of bacterial cells exposed to A-Ag-NPs, F-MWCNT, F-MWCNT-AgNPs Mueller-Hinton broth with different concentrations of A-Ag-NPs, F-MWCNT, F-MWCNT-AgNPs (0, 20, 40, and 60 μ L) was used, and the bacterial cell concentration was adjusted to 10^5 CFU/ml. Each culture was incubated in a shaking incubator at 37°C for 24 h. Growth curves of bacterial cell cultures were attained through repeated measures of the optical density (O.D.) at 600 nm.

3.7. Detection of reactive oxygen species

The reactive oxygen species (ROS) formed by F-MWCNT-AgNPs was identified using 2', 7'-dichlorofluorescein diacetate (DCFDA). The concentration of F-MWCNT-A-AgNPs treated was 40 μ L, and the number of bacterial cells used was adjusted to 10^5 CFU/ml. After all cultures were incubated at 37°C for 3 h, they were centrifuged at 4°C for 30 min at $300 \times g$, and then each supernatant was treated with 100 μ M DCFDA for 1 h. The ROS formed in the sample was detected at 475/20 nm of fluorescence excitation wavelength, and 521/20 nm of emission wavelength using Fluorescence Multi-Detection Reader (Xu et al., 2012).

3.8. Effect of F-MWCNT-A-AgNPs on protein leakage

Protein leakage from bacterial cells was detected using Bradford's protein assay. The concentration of F-MWCNT-A-AgNPs was adjusted to 40 μ L, and the concentration of bacterial cells was 10^5 CFU/ml. Each culture was incubated in a shaking incubator at 37°C for 6 h. 1 ml of culture sample was obtained from each culture (Dakal et al., 2016). The sample was centrifuged at 4°C for 30 min at $300 \times g$, and the supernatant was frozen at -

20°C. The supernatant was treated with Bradford's assay reagent, and the O.D. was measured at 595 nm.

3.9. Effect of F-MWCNT-A-AgNPs on respiratory chain LDH activity

The LDH activity was determined by measuring the reduction of NAD^+ to NADH and H^+ during the oxidation of lactate to pyruvate. In the second step of the reaction, diaphorase uses NADH and H^+ to catalyze the reduction of a tetrazolium salt to a highly colored formazan. The concentration of F-MWCNT-A-AgNPs was adjusted to 40 μ L, and that of bacterial cells was adjusted to 10^5 CFU/ml. Each culture was incubated in a shaking incubator at 37°C for 6 h. The culture was centrifuged at 4°C for 30 min at $300 \times g$, and the supernatant was discarded. The pellet was washed twice and then treated with LDH reaction solution in a microplate (Yuan et al., 2017). The plate was incubated with gentle shaking on an orbital shaker for 30 min at 37°C temperature. After incubation, the O.D. of the plate was determined at 490 nm.

3.10. Statistical analysis

All experiments were repeated at least three times. The results were represented as means $\pm$ SD. All experimental data were compared using Student's t-test. A p-value less than 0.05 was considered statistically significant.

4. Results

4.1. UV-Vis Spectroscopic

Fig.1 shows the UV-visible absorption spectrum of the AIE, $AgNO_3$, F-MWCNT, and F-MWCNTs-A-AgNPs, F-MWCNTs-A-AgNPs (20min and 40 min sonication) and F-MWCNTs-A-AgNPs in agar medium. The UV-visible scanning analysis of the product obtained displayed peaks and bands for SPR absorption. It was observed that the optimum reaction mixture for the best yield of the A-AgNPs was 1:7 ($AgNO_3$:AIE) ratios as at this point there

was highest band intensity, and peaks in SPR absorbance peak at 445 nm which is also clearly observed in F-MWCNTs-A-AgNPs in water and in agar medium. This data shows that the biological media to be used for the antibacterial studies did not change the stability of the A-AgNPs. The symmetric peak symbolizes that the solution does not contain any aggregated particles (Jha et al.,2017). The stability of the A-AgNPs was determined by measuring the absorption spectrum at 24-h intervals for 90 days. No significant changes in absorbance were determined during storage time at room temperature, indicating that the A-AgNPs were stable during this period. AgNO₃, F-MWCNT, F-MWCNTs-A-AgNPs solutions did not show any peak at 445 nm.

Figure-1: UV-Visible spectrophotometer of AIE, AgNPs, F-MWCNT (a), F-MWCNTs-A-AgNPs, F-MWCNTs-A-AgNPs for 20 min sonication, F-MWCNTs-A-AgNPs for 40 min sonication

4.2. XRD

In Fig.2, the XRD pattern of the A-AgNPs, F-MWCNT, F-MWCNTs-A-AgNPs and F-MWCNTs-A-AgNPs with sonication for 40 min are explained. The diffraction peaks for F-MWCNTs-A-AgNPs was found at 18⁰, 27⁰, 32, 35, 42⁰, 46⁰ and 52⁰, which is different from AgNO₃ and F-MWCNT with higher in intensity. The result was manipulated to study the crystal structure of A-AgNPs, and F-MWCNTs-A-AgNPs. Additionally after sonication, there was not changed diffraction peaks value of F-MWCNTs-A-AgNPs. The specific X-Ray diffraction pattern of the A-AgNPs

obtained from green rout was obtained by Bragg reflections corresponding to the (111), (200), (220), (311) and (222) sets of lattice planes are observed that may be indexed by the fcc structure of A-AgNPs (Jha et al., 2017; Ahmed et al., 2016).

Figure.2. XRD of A-AgNPs, F-MWCNT, F-MWCNTs-A-AgNPs and F-MWCNTs-A-AgNPs with sonication

4.3. FTIR

Fig.3 presents the FTIR spectra of the AgNO₃, A-AgNPs, F-MWCNT and F-MWCNTs-A-AgNPs. Importantly, the characteristic peaks seen for A-AgNPs alone are also evident in the sample of F-MWCNTs-A-AgNPs. By contrast, the performed peak at 1369 cm⁻¹ of AgNO₃ corresponding to the Ag group is not evident in the A-AgNP and peak at 1288 cm⁻¹ of A-AgNP is shifted in the F-MWCNTs-A-AgNPs sample (Ahmed et al.,2016).

Figure.3. FTIR of AgNO₃, AIE, AgNPs, F-MWCNT, F-MWCNTs-A-AgNPs and F-MWCNTs-A-AgNPs after sonication

4.4. TEM and DLS

TEM was presented on the F-MWCNT, and F-MWCNTs-A-AgNPs to visualize morphology of nanoparticles. The images of A-AgNPs was shown that particles are spherical and polydispersed with a size ranging from 40 to 60 nm in fig.4a. A-AgNPs was also crosslinked with F-MWCNT and presented surface of F-MWCNT in fig.4b. TEM examination was carried out at 1.5 Å resolutions, which is optimal for nanoparticle size analysis (Kang-ho et al 2011; Kumari et al., 2016). Next, DLS analysis exhibited that the formulated A-AgNP and F-MWCNTs-A-AgNPs had an average diameter of 132.4 ± 1.3 nm and 138.4 ± 1.4 .

Figure.4. TEM of A-AgNPs and F-MWCNTs-A-AgNPs

4.5. Growth curves of bacterial cells treated with different concentrations of A-Ag-NPs

The growth curves of bacterial cells treated with F-MWCNTs-A-AgNPs indicated that F-MWCNTs-A-AgNPs could inhibit the growth and reproduction of bacterial cells. The growth curves of F-MWCNTs-A-AgNPs treated *S. aureus* cells are shown in fig. 5(a). The bacterial growths of cells treated with 40 and 60 µg/ml F-MWCNTs-A-AgNPs were inhibited. After 4 h, almost all treated bacterial cells were dead. Similarly the growth curve of *E. coli* cells treated with F-MWCNTs-A-AgNPs is shown in fig. 5(b). These findings indicate that the antibacterial activity of F-MWCNTs-A-AgNPs could inhibit bacterial growth

but not enough to outpace the speed of reproduction of the bacterial cells. Interestingly, upon comparison of the bacterial growth curves, the growth curves of the F-MWCNTs-A-AgNPs - treated bacteria indicated a faster growth inhibition of *E. coli* than of *S. Aureus* (Pal et al., 2007).

Figure.5. Growth curves of *S. aureus* (A) and *E. coli* (B) cells exposed to different concentrations (0, 20, 40 and 60 µL) of F-MWCNTs-A-AgNPs

4.6. Formation of ROS from bacterial cells treated with F-MWCNTs-A-AgNPs

In this study, ROS was measured using DCFDA (Fig.6). After 3 h incubation, significantly increased ROS was detected in F-MWCNTs-A-AgNPs treated group of *S. aureus* or *E. coli* but not in control group. These results indicate that F-MWCNTs-A-AgNPs can form ROS with water, and so bacterial cell membrane, protein structure and intracellular system can be damaged owing to the ROS formed by F-MWCNTs-A-AgNPs.

Figure. 6. Formation of ROS in *S. aureus* (A) or *E. coli* (B) cells exposed to F-MWCNTs-A-AgNPs. The F-MWCNTs-A-AgNPs group was treated with F-MWCNTs-A-AgNPs at the concentration of 40 µL for 0 h or 3 h, and the control was not treated. **Significantly different from control group ($p < 0.01$).

4.7. Effect of F-MWCNTs-A-AgNPs on protein leakage from bacterial cell membranes

It was found that F-MWCNTs-A-AgNPs could enhance protein leakage by increasing the membrane permeabilities of *S. aureus* and *E. coli* cells (Fig. 7). Initially, protein leakage from the membranes of *S. aureus* cells treated with F-MWCNTs-A-AgNPs was almost the same as that from cells in the control group. At 6 h after incubation, protein leakage from cells treated with F-MWCNTs-A-AgNPs considerably increased; however, there was no change in the amount of protein leakage from cells in the control group. Leakage from cells treated with F-MWCNTs-A-AgNPs was significantly higher than that from cells in the control group.

Figure.7. Leakage of protein from *S. aureus* (A) or *E. coli* (B) cells exposed to F-MWCNTs-A-AgNPs (concentration of 40μL and the control was not treated)

*,**Significantly different from control group (*: $p < 0.05$; **: $p < 0.01$)

4.8. Effect of F-MWCNTs-A-AgNPs on respiratory chain lactate dehydrogenase activity

To determine oxidative stress-induced damage of the respiratory system of the cells, LDH activity was measured. The effects of F-MWCNTs-A-AgNPs on LDH activities of *S. aureus* and *E. coli* cells are shown in fig. 8. The LDH activity of *S. aureus* cells in the control group increased with time, whereas the LDH activity decreased slightly in the cells treated with Ag-NPs. LDH activity in cells treated with F-MWCNTs-A-AgNPs was significantly lower than that in cells of the control

group. Moreover, the LDH activity of *E. coli* cells in the control group increased considerably with time, whereas that of the cells treated with F-MWCNTs-A-AgNPs decreased slightly; there was a significant difference in the LDH activities in F-MWCNTs-A-AgNPs treated cells and in cells of the control group. These results indicate that ROS formed by F-MWCNTs-A-AgNPs inhibit LDH, an important enzyme in cellular respiration. As a result, F-MWCNTs-A-AgNPs cause inhibition of bacterial growth and reproduction, in agreement with a previous study, which showed that the respiratory chain activity in *E. coli* was inhibited by F-MWCNTs-A-AgNPs (Yildirimer et al., 2011).

Figure. 8. Effect of F-MWCNTs-A-AgNPs on respiratory chain lactate dehydrogenases in *S. aureus* (a) or *E. coli* (b) (concentration of 40μL, and the control was not treated)

**Significantly different from Control group ($p < 0.01$).

5. Discussion

Several lines of evidence suggest that AgNPs have a wide variety of applications in diverse fields, including antimicrobial activities. Recently, investigators reported that F-MWCNT-A-AgNPs are capable of antimicrobial activities in different purposes in vitro (Engering et al., 2013). However, there is a lack of literature regarding the use of AgNPs as antibacterial agents against bacterial cell (Zhang et al., 2016). Moreover the detailed biochemical mechanisms of the antibacterial activity of F-MWCNT-A-AgNPs using plant extract were not known. In the present report, we

have shown for the first time that F-MWCNT cross linked with A-AgNPs exhibit antibacterial activity in two bacterial strain *S. aureus* and *E. coli* cells using plant extract and determined the mechanism of action. This study has systematically established the antibacterial activity of F-MWCNT-A-AgNPs. For this study, we prepared a stable purified form of A-AgNP using *Azadirachta indica* leaf extract as a reducing agent and cross linked with functionalized MWCNT which showed remarkable stability and uniform distribution throughout the test period without any sign of aggregation and precipitation fig.1 to fig.4. Thorough physiochemical characterization of the F-MWCNT-A-AgNPs revealed excellent average size and uniform surface charge.

In summary, we have synthesized and characterized a series of F-MWCNTs-A-AgNPs complexes. The antibacterial activities of F-MWCNTs-A-AgNPs were studied with respect to Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* by observing the bacterial cells treated or not with F-MWCNTs-A-AgNPs by measuring the growth curves, formation of bactericidal reactive oxygen species (ROS), protein leakage, and lactate dehydrogenase activity involved in the respiratory chain. Bacterial cells were treated with F-MWCNTs-A-AgNPs, and the growth rates were investigated under varying concentrations of different type of nanocomposites. As a result, *S. aureus* and *E. coli* were shown to be substantially inhibited by F-MWCNT, AgNPs, F-MWCNTs-A-AgNPs, and the antibacterial activity of F-MWCNTs-A-AgNPs did not fluctuate with temperature or pH. These results suggest that F-MWCNTs-A-AgNPs could be used as an effective antibacterial material (Vardharajula et al., 2012; Liangpeng et al., 2014; Niu et al., 2009; Xue et al., 2008).

Based on the above observations we have drawn a scheme of the mechanism of action and of F-MWCNTs-A-AgNPs in bacterial cell (Fig.9). Hybrid F-MWCNTs-A-AgNPs act on several pathways but the major action is through chain lactate dehydrogenase, protein leakage and ROS. Importantly, the data presented here from the antimicrobial activities of F-MWCNTs-A-AgNPs suggest cell death in bacterial cell.

Figure 9. The mechanism of action of fabrication and antimicrobial activities of F-MWCNTs-A-AgNPs.

6. Conclusion

The study involved the green method for the production of A-AgNPs using the leaf extract of *Azadirachta indica*. The major reaction involved in the formation of the nanoparticles is the reduction of the carboxylic acid groups. The work also describes the synthesis and surface functionalization of A-AgNPs and F-MWCNTs-A-AgNPs. The results of the AgNPs synthesis and their functionalization were checked by using XRD, FTIR, TEM, DLS, and UV/Vis spectroscopy techniques. A-AgNPs exhibited low antibacterial activity, which is significantly increased by the F-MWCNTs-A-AgNPs. Light triggering of these functional F-MWCNTs-A-AgNPs further increases their efficiency in the killing of both gram-negative and gram-positive bacteria. The present method is

free of such weaknesses, and it gives a favourable opportunity to the creation of functional green F-MWCNTs-A-AgNPs for in vivo and other medical applications.

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