Synthesis of Cu/CNTs nanocomposites for antimicrobial activity

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Abstract

We report a facile method for the synthesis of Cu/multi-walled carbon nanotubes (CNTs) composite powder employing a chemical reduction method followed by high-energy ball milling involving the use of sodium borohydride as a reducing agent and copper sulphate as the precursor material. Control of oxidation of Cu nanoparticles (CuNPs) is a key factor in the synthesis of Cu/CNTs nanocomposites via chemical reduction methods and other methods. To overcome this problem we have applied a new facile rapid synthesis method using a combination of molecular-level mixing followed by high-energy ball milling to produce mostly CuNPs. X-ray diffraction results indicated the presence of mostly CuNPs in composite powder. Scanning electron microscopy and high resolution transmission electron microscopy (HRTEM) was used to ascertain the dispersion of CNTs in Cu matrix. Most of the CuNPs synthesized in the present work had a particle size ranging from 20–50 nm as revealed by HRTEM characterization. Moreover, the CNTs were also found to be homogeneously dispersed in Cu matrix. The Cu/CNTs nanocomposite has a wide range of applications from fuel cells to electronic chip components. In the present work we have investigated the antimicrobial activity of Cu powder and varying concentrations of Cu/CNTs nanocomposite against gram negative Providencia sp. bacteria, and gram positive Bacillus sp. bacteria. These findings suggest that Cu/CNTs nanocomposite can be used in antibacterial controlling systems and as an effective growth inhibitor in the case of various microorganisms.

Keywords: carbon nanotubes, copper nanoparticles, XRD, SEM, HRTEM, antimicrobial activity

Classification numbers: 5.11, 5.14

1. Introduction

Synthesis and applications of Cu/CNTs nanocomposites is a field of active research [1–3]. However, one of the major challenges in the production of Cu/CNTs composites is to prevent the oxidation of Cu nanoparticles [1, 3, 4]. Many researchers have prepared Cu/CNTs composites using a number of methods such as molecular-level mixing [1, 5], chemical reduction methods [6–11], thermal reduction [12], microemulsion technique [13], polyol method [14] and dc and arc discharge [15] etc. Although significant efforts have been made to synthesize pure CuNPs, it has been observed that copper nanoparticles synthesized at ambient P–T conditions inevitably had a thin layer of oxide on their surface because the Cu oxide phases are thermodynamically more stable than pure Cu. Also, copper nanoparticles are found to aggregate severely without proper protection. Thus, some of these methods [1, 9, 11] resulted in the formation...
of a small concentration of CuO/Cu2O along with CuNPs as confirmed by x-ray diffraction (XRD) and high-resolution transmission electron microscopy (HRTEM). Reduction of the CuO/Cu2O/CNTs composite powder has been performed by heating the mixture in a hydrogen atmosphere; however, this reduction method has not been elucidated and details remain ambiguous [1]. Where this reduction process has been elucidated, it involves the use of H2 gas at 400 °C [5, 16]; however, the effect of heat treatment at 400 °C on the particle size of obtained CuNPs has not been made clear, as it is believed that at higher temperatures, grain growth in particle size may occur. Microorganisms have become a major obstacle in everyday life. With increasing populations and stress on existing resources, microbial infections are on the rise every day, resulting in up-scaling of medical costs. Increasing resistance of microbes to multiple antibiotics and drugs has become an issue of concern. Considering all these factors, it is imperative to develop alternative systems of microbial control. Researchers have established the role of Ag nanoparticles in the control of microbial infections [17–19]. Carbon nanotubes have become an area of advanced research since their discovery [20]. One of their major applications is antimicrobial activity, which is an important area of current research [21, 22]. The antimicrobial activity of single-walled carbon nanotubes (SWCNTs) has recently been reported [23]. It is proposed that the cell wall is damaged due to interaction with pristine SWCNTs. Multi-walled carbon nanotubes (CNTs) have also displayed antimicrobial activity, though they are inferior to SWNTs [23]. Increasingly, the role of Cu nanoparticles pertaining to antimicrobial activity has also been researched [21, 24–26]. Although copper nanoparticles have demonstrated higher bactericidal activity against Bacillus subtilis than silver nanoparticles [24], the antimicrobial activity of copper nanoparticles is yet to be fully ascertained. In today’s demanding situation it is important to develop composites of these materials for a synergistic effect against microbes. Much of the work till now regarding the use of copper nanoparticles as antimicrobial agents has been done on Escherichia coli [24, 25, 27]. In the present study, we have investigated the role of copper/multi-walled carbon nanotube nanocomposites (Cu/CNTs) antimicrobial activity on Providencia sp. and Bacillus sp. We chose to perform the antimicrobial studies on Bacillus sp., a gram positive rod-shaped bacterium, as they are a major cause of food spoilage and also cause food-borne illnesses. Curbing the effects of this bacterial species would not only be beneficial industrially but would also help in preventing illnesses. Providencia is a gram negative motile bacterium and opportunist pathogen, causing urinary tract infections in patients with long-term usage of catheters or extensive burns. Providencia were isolated from 18% of complicated urinary tract infections [28]. Providencia infections with antimicrobial resistance patterns are increasing [29] giving impetus for ushering in better remedial systems. Study of antimicrobial activity of CuNPs on Bacillus is limited, and has never been carried out on Providencia species, and hence this has been investigated in our current work. The basis of bactericidal effect of metal nanoparticles is on their small size and high aspect ratio which allows them to have a better interface with bacterial cell walls [30]. It is beneficial to study the antimicrobial effect of copper nanoparticles as they are low-cost compared to silver or gold nanoparticles. Copper nanoparticles will become more important, as they could be an essential part of nanodevices due to their excellent conductivity and good biocompatibility [31]. The study of metal ions as antimicrobial agents is important because of their broad-spectrum antimicrobial activity and their absence of cross-reactivity with antibiotics [32]. Based on enhanced effectiveness, the new age drugs are nanoparticles of polymers, metals or ceramics, which can combat conditions like cancer [33] and fight human pathogens like different bacteria [30, 34–37]. We envisage that Cu/CNTs composite materials could be used as thin films on catheter linings, hospital trays, in food containers, or on any surface which is liable to microbial infection, so as to stem the infection caused by microbes. Although multi-walled CNTs are somewhat inferior in quality in comparison to SWCNTs, their antimicrobial activities against both the gram positive and gram negative are as prominent as those observed previously using SWCNTs. Further, CNTs are much cheaper as compared to SWCNTs and, therefore, the present work on the bactericidal effect of nanocomposite of Cu/CNTs is not only very effective but also economical. The antimicrobial effect of Cu/CNTs on gram positive Bacillus sp. and gram negative Providencia sp. have been described in detail in this paper for the first time to the best of our knowledge.

2. Experimental

2.1. Materials

The starting materials used in the present investigation were copper sulphate, ethylenediaamnine tetracetic acid (EDTA), sodium borohydride (NaBH4), sodium dodecyl sulphate (SDS), sodium hydroxide (NaOH) (all purchased from M/s Fisher Scientific Inc), and multi-walled carbon nanotubes (Nanocyl, Belgium, diameter 15–25 nm, length 1.5 μm with specific area of 250–300 m2 g−1). Bacterial strains were provided by Indian Agricultural Research Institute (IARI), New Delhi. The bacterial strains were isolated and cultured from root rhizospheres of indigenous plants grown in the fields of IARI and other various parts of India. The bacterial strains were identified by using molecular chronometer 16S rRNA gene sequencing tool, commonly used to study the bacterial phylogeny and taxonomy. All these strains had been submitted to National Center for Biotechnology Information (NCBI) with accession number HM452309 (Bacillus sp.) and FJ866760 (Providencia sp.). For all experiments the nanoparticles suspensions were dispersed in milli-Q water and sonicated for 5 min in a water bath sonicator at 40 ± 3 kHz (Model EN-100-US; ‘Fast-Clean’ Ultrasonic Cleaner. Life Care Equipments Pvt Ltd).

2.2. Functionalization of CNTs

The functionalization of CNTs can be done using various methods such as ball milling [38], plasma treatment [39] and acid treatment [40]. In the present work, acid functionalization of multi-walled CNTs was done using a mixture of H2SO4 and HNO3 in 3 : 1 ratio. CNTs were refluxed in the acid mixture for 10 h at 80 °C. Subsequently, the CNTs were dried in a
vacuum oven at 80°C. This results in the creation of –COOH and –OH groups on the surface of the CNTs, thus providing a bonding site for the metal matrix to the CNTs and ensuring a highly dispersed system.

2.3. Synthesis of Cu/CNTs nanocomposite powder

Various precursor materials can be used for the synthesis of copper nanoparticles. CuSO₄ was used in the current work. CuSO₄, NaOH, SDS and EDTA are taken in distilled water. EDTA was employed as an oxidation control agent, to avoid the formation of copper oxide species. Metal nanoparticles are generally prone to oxidation due to higher surface area and smaller size. NaOH was added to the solution to maintain the pH around 10–11; also the reaction proceeds at a faster rate. SDS acts as a surfactant and ensures the homogeneous dispersion of CNTs in aqueous solution. The solution was kept under magnetic stirring at 50°C. A deep blue colour was observed due to the dissolution of CuSO₄. Acid functionalized CNTs were added to the solution. NaBH₄ was added as a reducing agent. Immediately on the addition of NaBH₄ a colour change was observed from deep blue to dark red, which indicated the formation of copper nanoparticles. Materials were used in the following concentrations 1 M EDTA, 0.04 M CuSO₄, 0.1 M NaOH, 0.25 M NaBH₄; CNTs were added as required. EDTA and SDS were selected as these reagents are biologically benign. In fact, EDTA is used to cure lead and heavy metal poisoning, known as chelation therapy. SDS is normally used as a surfactant. It has shown antiviral and antimicrobial activity, and hence we employed it here [41, 42]. Hence, materials were selected on dual basis.

Cu/CNTs composite powder produced by the above-mentioned method was further ball milled at 250 rpm for 2 h keeping ball-to-powder wt. ratio at 10:1. Ethanol was used as a process control reagent to avoid any cold annealing during the milling process. Ball milling results in further lowering of the particle size, and more importantly it helps in grain refinement of the material which improves the interface between the CNTs and Cu nanoparticles.

2.4. Antimicrobial tests

2.4.1. Disk diffusion test. Antimicrobial activity of antibiotics is normally tested using a disk diffusion test, employing antibiotic infused disks [43]. A similar test with nanoparticles instead of antibiotics was performed with a little change in this study. For unicellular bacterial system, a culture of gram negative Providedencia sp. and another of gram positive Bacillus sp. were grown overnight on a nutrient broth medium at 28 ± 2°C to provide optimum temperature for growth in a rotary shaker at 150 rpm to provide proper aeration, as these are aerobic microbes. The aim for maintaining this condition was to obtain microbes at a concentration of 10⁶ colony forming units per 1 ml (CFU/ml) and to check clump formation of cells among them. The cultures (100 μl) of 10⁶ CFU/ml were uniformly plated on nutrient agar (NA) plates, and three wells (2 mm diameter) were made with the sterile core borer. The aqueous solution of Cu/CNT composites and pure copper of 100 μl each (1 mg ml⁻¹) were loaded to the wells and the plates were incubated at about 28 ± 2°C. After ~48 h of incubation, there resulted a zone of inhibition on the plates. The average diameter of the inhibition zone (DIZ) surrounding the disks was measured to determine inhibition.

2.4.2. Broth culture test. Broth culture tests help in overcoming some of the shortcomings of disk diffusion tests and hence can be used to confirm the result of disk diffusion tests. Disk diffusion as the name suggests, depends on the diffusion of the antimicrobial agent through the nutrient agar medium. Carbon nanotubes can aggregate inside the pores of the agar and may prevent diffusion and hence may give false results, it is imperative therefore to establish results only after carrying out broth culture tests. Providedencia sp. and Bacillus sp. were grown in 50 ml of nutrient broth, overnight in a shaker incubator (28 ± 2°C, 150 rpm), resulting in a broth of logarithmic phase microbes containing 10⁶ CFU/ml as prepared formerly for disk diffusion test. Side-armed flasks were prepared with 25 ml of nutrient broth. Around 10% bacterial culture and 10% (5 μg ml⁻¹) Cu/CNTs nanocomposites were inoculated to this. Flasks were kept in a shaker incubator (28 ± 2°C, 150 rpm). Control broths in two side-armed flasks were used containing gram positive and gram negative microbes without nanoparticles. Growth was monitored by obtaining measurements of the optical density (OD) at 620 nm (visible range); that is, an initial reading was taken at zero hour in a colorimeter and subsequently readings were taken in each two hour interval up to 12 h in six lots.

2.5. Instrumentation

A scanning electron microscope (SEM, model LEO 440) equipped with an energy depressive spectrometer (EDS, model Oxford Link ISIS 300) was used to study the microstructure of the Cu/CNTs nanocomposite powder. A FEI model Tecnai G2 F30 STWIN, 300 kV machine was used to carry out HRTEM of Cu/CNTs nanocomposite powder produced in this work. X-ray diffraction tests were performed at room temperature on a Rigaku MiniFlex™ II system using CuKα radiation (λ = 0.15418 nm). A colorimeter was used to study the OD of the broth cultures. Rotary shakers were used for the culture of bacteria and for the broth culture tests. UV–visible tests were conducted on a Perkin-Elmer series spectrophotometer.

3. Results and discussion

3.1. X-ray diffraction analysis

Figure 1(a) shows the XRD pattern for Cu/CNTs synthesized using 0.1 M EDTA. As can be seen, there is the presence of a high quantity of Cu₂O species and a lower quantity of Cu. Oxidation of Cu nanoparticles ensues directly after their formation because nanoparticles are highly reactive due to increased surface area. In this work we have used EDTA as an oxidation control agent; it prevents the oxidation of Cu nanoparticles in solution. 0.1 M EDTA is not sufficient in preventing oxidation of Cu nanoparticles. Higher concentrations of EDTA were tried. Figure 1(b) shows the XRD pattern of Cu/CNTs produced by using 1.0 M EDTA,
this concentration of EDTA is proficient in avoiding the oxidation of most of the Cu nanoparticles and hence was used in the current work to produce Cu/CNTs composite powder. No contamination or unwanted materials were seen in the X-ray diffraction results. Copper nanoparticles are present in their pure form and not as cuprite particles.

From the XRD results shown in figures 1(a) and (b), it is seen that a higher concentration of EDTA (1.0 M) favours the formation of Cu nanoparticles. Samples obtained without using EDTA showed XRD peaks mostly due to Cu$_2$O (figure 1(c)). With the addition of 0.1 M EDTA, the product contained mostly Cu$_2$O along with Cu nanoparticles as shown in figure 1(a). However, by increasing the concentration of EDTA to 1.0 M, the XRD peaks due to Cu$_2$O almost disappeared, leaving the formation of Cu nanoparticles. The effect of EDTA addition on the formation of Cu nanoparticles has also been studied earlier [44] using a small concentration of EDTA. In these experiments, although the size of Cu nanoparticles was around 4 nm, the yield was very small and the product contained a higher concentration of Cu$_2$O instead of pure Cu nanoparticles, as revealed by XRD results. However, in the present work, using 1.0 M EDTA, we observed that the peaks due to Cu$_2$O were almost absent in the XRD pattern.

Addition of NaOH solution to CuSO$_4$ generally leads to the formation of a blue colour due to Cu(OH)$_2$. In order to produce Cu nanoparticles, NaBH$_4$ was added as a reducing agent in a basic environment together with EDTA. The reducing process for the formation of Cu nanoparticles in the presence of EDTA is shown in the following equations:

\[
\text{CuSO}_4 + 2\text{NaOH} \rightarrow \text{Na}_2\text{SO}_4 + \text{Cu(OH)}_2, \\
\text{Cu(OH)}_2 + \text{EDTA} \rightarrow \text{Cu-EDTA} + 2\text{OH}^-, \\
\text{Cu-EDTA} + 2\text{NaBH}_4 + 2\text{OH}^- + 2\text{H}_2\text{O} \rightarrow \text{Cu} + 2\text{NaBO}_2 + 7\text{H}_2 + \text{EDTA}.
\]

The regenerated EDTA as shown in equation (3) again reacts with unconverted Cu(OH)$_2$ and the process repeats until the whole amount of NaBH$_4$ is consumed, as shown in equation (3). Therefore, although a small concentration of EDTA is sufficient for the formation of Cu nanoparticles, its amount should be adequate enough to convert all Cu(OH)$_2$ into Cu-EDTA, as shown by equation (2). Lower concentration of EDTA leads to the formation of only trace amount of Cu atoms, as observed earlier [44].

3.2. Characterization of functionalized CNTs (fCNTs)

Surface-modified CNTs prepared in the present work were also characterized by measuring the Zeta potential at different pH values. Although fCNTs were dispersed easily in distilled water, pure CNTs could not be easily dispersed. Therefore, pure CNTs as well as fCNTs were dispersed in 10 mM NaCl solution and the pH of the solution was adjusted by using 0.1 M HCl followed by ultrasonication for more than 10 min. For pH 6 only distilled water was used. The results of zeta potential measured for pure and fCNTs at different pH values are shown in table 1.

From table 1, it is seen that a more negative value of zeta potential was observed for fCNTs at all pH values. However, a positive value of zeta potential was observed for pure CNTs. This change in zeta potential values might be attributed to the surface modification of CNTs, which
Table 1. Zeta potential of pure CNTs and fCNTs (in mV)

| pH | Pure CNTs | fCNTs |
|----|-----------|-------|
| 2  | 27.9      | -9.48 |
| 3  | 25.7      | -40.4 |
| 4  | 8.12      | -40.6 |
| 5  | 1.43      | -54.5 |
| 6  |           | -43.6 |

Figure 2. Raman spectra of (a) pure CNTs and (b) functionalized CNTs.

introduces COO\(^-\) groups on to the surface of CNTs. Acid functionalization introduced electrical repulsive charges on CNTs. A carboxylated nanotube will ionize in polar solvents and the repulsive forces between COO\(^-\) groups will hold the CNTs apart, preventing their agglomeration. The zeta potential of fCNTs has a more negative value than pure CNTs, as shown in table 1, thus confirming their higher degree of dispersion. Similar results were obtained earlier [37].

Functionalized CNTs produced in this work were also characterized using Raman spectroscopy. Raman spectroscopy is a powerful non-destructive tool to characterize carbonaceous materials, particularly for distinguishing ordered and disordered crystal structure of carbon. The typical features of carbon in Raman spectra are the G band (graphite) around 1582 cm\(^{-1}\) and the D band (defect) around 1350 cm\(^{-1}\). The G band is usually assigned to the E\(_{2g}\) photons of C sp\(^2\) atoms, while the D band is a breathing mode of k-point phonons of A\(_{1g}\) symmetry.

Figures 2 shows the Raman spectra of pure CNTs and fCNTs used in the present work. The Raman spectra were measured using 514.5 nm laser excitation over the Raman shift interval of 1000–2000 cm\(^{-1}\). The D- and G-bands of pure CNTs at around 1290 and 1590 cm\(^{-1}\), corresponding to defect- and disorder-induced modes, and the in-plane E\(_{2g}\) zone centred mode, are clearly observed in pure CNTs.

3.3. Fluorescence spectroscopy of copper nanoparticles

Splitting of energy levels due to the quantum size effect [45] becomes more distinct as nanoparticles become smaller in size. Energy level splitting provides an abundance of electronic transitions in nanoparticles. Surface plasmon resonance (SPR) is not available at the nanoscale, for dissipation of incident energy, so transition takes place from discrete valence states to excited valence states in the case of Cu nanoparticles [45]. 3d valence and 4sp conduction electrons are responsible for fluorescence pertaining to Cu nanoparticles. On excitation with a 275 nm wavelength there is excitation of d-band electrons into the sp-conduction band. This shift in electrons from a lower valence state to a higher valence state and their subsequent recombination into the original states leads to creation of fluorescence, this occurs at roughly 480 nm [46–48]. The photoluminescence (PL) spectra of Cu nanoparticles obtained with a 250 nm Xe laser excitation at room temperature using 0.1 M EDTA and 1.0 M EDTA are shown in figures 3(a) and (b), respectively.

From these spectra it is seen that a sharp PL peak is observed at 375.5 nm, when EDTA of 0.1 M concentration was used and a slight shift in PL emission peak (264 nm) is observed when 1 M EDTA was used. The shift in PL emission peak in two cases can be attributed to the stochiometric defects due to the excess concentration of EDTA in the solution. In general, the particle size confinement results in the shift in wavelength. Increase in particle size shows a red shift in PL emission. The increase in grain size of Cu nanoparticles using a higher concentration of EDTA (1 M) has been confirmed by XRD results shown in figures 1(a) and (b), respectively. Using the Scherrer’s equation, the average grain size of Cu nanoparticles synthesized using 0.1 M EDTA was calculated to be 21.3 nm and those synthesized using 1.0 M EDTA is about 40 nm.
3.4. UV–vis spectra of Cu nanoparticles

Colloidal dispersion of metal particles exhibits absorption spectra in the UV–vis region. Absorption in metal particles is mainly caused by the excitation of SPR. SPR is due to the collective oscillation of free electrons of the metal nanoparticles in resonance with the frequency of incident light wave \([49–51]\). Nanosized Cu nanoparticles with size larger than 20 nm typically exhibit a SPR peak at 560 nm \([52, 53]\).

Figures 4 represents the UV–vis spectra of Cu nanoparticles synthesized using 0.1 M EDTA and 1.0 M EDTA in the present work. In both cases a broad absorption peak in the UV–vis spectra is obtained at 287.5 nm (using 0.1 M EDTA) and 297.5 nm (using 1.0 M EDTA). As in both cases, the UV–vis spectra are obtained at wavelengths lower than 300 nm, indicating that the size of the copper nanoparticles synthesized in the present work is less than 50 nm \([54]\). A broad peak around 300 nm in the UV–vis spectra also suggests that the size of the majority of the Cu nanoparticles is uniform and varied in the small range of 20–50 nm.

3.5. Characterization of Cu/CNTs nanocomposites using SEM and HRTEM

Figure 5(a) represents the SEM and figure 5(b) represents the HRTEM images showing the microstructure of Cu/CNTs composites fabricated in the present work. CNTs can be seen dispersed homogeneously throughout the Cu matrix. A highly dispersed system with minimal agglomeration of CNTs is essential for the Cu/CNTs composite to have optimal qualities. Better dispersion aids in the formation of an optimal interface between the Cu matrix and CNTs, and the interfacial bonding between Cu and CNTs is enhanced via good dispersion of CNTs.

3.6. Antimicrobial activity of Cu/CNTs

The main aim of this work was to study the antimicrobial activity of Cu/CNTs nanocomposites. We have employed two methods to attain accurate results, viz disk diffusion test and broth culture test. Gram negative (Providencia sp.) and gram positive bacteria (Bacillus sp.) were taken for the antimicrobial tests. Disk diffusion tests are normally employed for testing antimicrobial activity of antibiotics. The main disadvantage with disk diffusion tests in the case of nanoparticles is that they may agglomerate inside the agar medium and diffusion will not take place. This will give a false-negative result, especially as in the case of CNTs the chance for blockage of pores of agar medium is high. A broth culture analysis is required to double-check the results, avoiding any false negatives which present in disk diffusion tests.

Disk diffusion, as the name suggests, works on the following principle: as the antimicrobial agents diffuse through the agar medium (in this case Cu/CNTs nanocomposite), they express their antimicrobial activity
Figure 6. Disk diffusion test of (a) *Providencia* sp. and (b) *Bacillus* sp.

Figure 7. Broth culture test of (a) *Providencia* sp. and (b) *Bacillus* sp.

and an inhibition zone is seen. The inhibition zone is indicative of the antimicrobial activity of the substance being tested as there is no growth of microbes in this zone. The diameter of inhibition zone is greater for bacteria which are highly susceptible to the antimicrobial agent being tested, and smaller for bacteria which show comparatively greater resistance. Zones of inhibition were measured using HiAntibiotic Zone Scale (HiMedia, Mumbai, India), there is some scope for error but in general the results are accurate.

Figure 6(a) shows a disk diffusion test for *Providencia* sp. against pure Cu, Cu/CNTs (1 wt% CNT) and Cu/CNTs (10 wt% CNTs). Nearly negligible activity is observed in this case, which is due to the fact that *Providencia* sp. is an extremely fast-growing species and the rate of diffusion for the Cu/CNTs is relatively slower, giving a false impression that there is minimal bactericidal activity. Figure 6(b) illustrates the bactericidal effect of pure Cu, Cu/CNTs (1 wt% CNTs) and Cu/CNT (10 wt% CNTs) against *Bacillus* sp. The inhibition zones can be clearly seen from figure 6(b). The diameter of zone of inhibition is 26 mm for Cu/CNT (1 wt%), 18 mm for Cu/CNTs (10 wt%) and 15 mm for Cu (pure). According to these results the bactericidal effect of Cu/CNTs (1 wt%) is the maximum, followed by Cu/CNTs (10 wt%) and finally Cu (pure). In the case of nanoparticles these results cannot be considered conclusive due to diffusion complications.

Figures 7(a) and (b) show broth culture test samples of *Providencia* sp. and *Bacillus* sp., respectively, and their results are summarized graphically in figures 8 and 9.

From these graphs it is evident that there is bactericidal effect of Cu/CNT present in microbial broth cultures, confirming that a false negative was obtained in the disk diffusion test of *Providencia* sp. The broth culture analysis was done for 12 h to insure that obtained results were coherent over a long time. After measurement of optical density (OD) for 12 h, the cultures were left undisturbed for 24 h and the OD was measured again to account for any changes in bacterial growth over a long period. The OD after 24 h was similar to 12 h in all test samples of Cu/CNTs. Broth culture analysis is a better indicator of bactericidal effect as the colloidal nanocomposite particles are in direct contact with the bacteria at all times; exposure of bacteria to the nanoparticles is maximum because of continuous agitation given at 150 rpm. The OD is a measure of the turbidity of a solution, also known as absorbance in terms of spectroscopy, and it follows the Beer–Lambert’s law. In terms of microbiology the OD is indicative of the turbidity of a solution and can be used to estimate the number of bacterial cells in a solution. A high absorbance is reflective of a high number of bacteria present in a test solution and a lower absorbance reflects a lower concentration of bacteria. Optical density measurements are taken on a colorimeter. For calibration, a control solution is used as a base line (control) for the measurement of test solutions. It can be seen from the graphs that both bacterial species are highly susceptible to the Cu/CNTs mixture in comparison to pure Cu. It was interesting to note that in the case of Cu/CNTs (10 wt%) a rise in the concentration of bacteria is observed before the killing of bacteria begins and a high absorption was observed initially because of black
Figure 8. Bactericidal effect of (a) Cu/CNTs (1 wt%), (b) Cu/CNTs (10 wt%) and (c) pure Cu over 12 h period on Providencia sp.

colour due to CNT particles. This phenomenon takes place possibly because concentration of CNTs is quite high, and the Cu/CNTs nanocomposite is dissolved in the nutrient medium, and hence the dispersion of Cu/CNTs (10 wt%) could be minimal at initial stages, and therefore the growth of bacteria is observed. When a homogeneous dispersion is observed with respect to time and agitation, the OD drops significantly, showing the onset of bactericidal effect of Cu/CNTs. Lower absorbances in initial stage and final stage are observed in the case of Providencia sp., and this indicates that the specie is more susceptible to the Cu/CNT than Bacillus sp. The killing rate of bacteria can be estimated using the change in OD, if the initial concentration of OD is taken to be that in the presence of 100% bacteria, and final OD is taken to be that at the final concentration of bacterial cells in solution. Negative absorbance can be seen in some cases. This can be due to slight error in the machine, and also because copper nanoparticles show fluorescence when excited. As the OD approaches zero it is possible that the incident wavelength (620 nm) causes fluorescence of copper nanoparticles, giving negative absorbance. But in the case of Cu/CNTs (1 wt%) due to lower percentage and proper dispersion, bactericidal effect was amazingly effective. The absorbance recorded initially at zero hour is high because of black colour due to CNT particles and goes down as the time increases. In both cases, with Providencia and Bacillus, the OD bactericidal effect of pure Cu is as prominent as that of Cu/CNTs (10 wt%), as can be seen in figures 8 and 9. The killing rate was estimated using the following formulae:

\[
\text{Killing rate (\%)} = \frac{C_{\text{init}} - C_{\text{final}}}{C_{\text{init}}} \times 100\%,
\]

\[
C_{\text{final}} = \frac{(\text{Final OD})}{(\text{Initial OD})} \times C_{\text{init}},
\]

where \(C_{\text{init}}\) is initial concentration of bacteria and \(C_{\text{final}}\) is final concentration of bacteria.

Table 2 represents the killing rates of different antimicrobial test agents. It is evident from this table that pure copper (µm size powder) displays no bactericidal effect. Providencia sp. is highly susceptible to Cu/CNTs; the addition of CNTs increases the killing rate of the bacteria. Bacillus sp. is more resistant to the Cu/CNTs nanocomposites compared to Providencia sp. Increase of the concentration of CNTs actually inhibits the bactericidal effect of nanocomposite against Bacillus sp. More work is needed in this regard with special focus on elucidating the mechanism of antibacterial activity. Looking at the results, it can be established that gram positive Bacillus sp. is less susceptible to Cu/CNTs mixture and Providencia sp. is highly susceptible to Cu/CNTs mixture.

The exact mechanism of bacterial cell death by copper nanoparticles is yet to be established. Release of ions in solution can lead to bactericidal effect [55]. Metal depletion
Figure 9. Bactericidal effect of (a) Cu/CNTs (1 wt%), (b) Cu/CNTs (10 wt%) and (c) pure Cu over 12 h period on Bacillus sp.

Table 2. Killing rate of bacteria by different concentrations of Cu/CNTs and pure Cu.

|        | Providencia         | Bacillus          |
|--------|---------------------|-------------------|
| (%)    | (%)                 | (%)               |
| Cu (Pure) | 0.29               | 0.1               |
| Cu/CNTs (1 wt%) | 86.07             | 58.32             |
| Cu/CNTs (10 wt%) | 99.11            | 38.89             |

may cause the formation of irregularly shaped pits in the outer membrane and change membrane permeability, which is caused by progressive release of lipopolysaccharide molecules and membrane proteins [56]. The basic differences between gram positive and gram negative bacteria essentially rest in the structure of their respective cell walls. The gram negative bacteria have a layer of lipopolysaccharide at the exterior, followed underneath by a thin (around 7–8 nm) layer of peptidoglycan [57]. Although the lipopolysaccharides are composed of covalently linked lipids and polysaccharides, they lack strength and rigidity. On the other hand, the cell wall in gram positive bacteria is principally composed of a thick layer (about 20–80 nm) of peptidoglycan, consisting of linear polysaccharide chains cross-linked by short peptides to form a three dimensional rigid structure [58].

It is difficult to distinguish between the effect of ions released by nanoparticles and that of nanoparticles themselves [59]. Due to presence of negative charges on the lipopolysaccharides [60], it is possible that the Cu nanoparticles get attached to the negatively charged bacterial cell wall and cause its lysis, leading to bacterial cell death [60]. The bactericidal effect of Cu/CNTs as a nanocomposite is yet to be elucidated and more work is needed in this direction.

4. Conclusion

Studies on the bactericidal effect of Cu/CNTs with varying concentration of CNTs against different bacteria were conducted. It is found that gram negative bacteria Providencia is more susceptible to Cu/CNTs nanocomposites as compared to gram positive bacteria Bacillus. We have reported MICs which are comparable and lower than in already reported work. This study shows that Cu/CNTs have great potential for antimicrobial applications. It is safe to assume that copper nanoparticles have great affinity to the surface groups of bacterial cell walls, and carbon nanotubes increase the interface area between the Cu/CNTs nanocomposite and bacterial cell walls. Due to the high susceptibility of Providencia sp. it is possible to develop Cu/CNTs to combat bacterial infections caused by this species in clinical settings, Cu/CNTs thin films would be efficient in minimizing the effect of bacteria. Cu/CNTs thin films could be used on catheter linings, surgical instrument surfaces, hospital furniture surfaces etc. Bacillus sp. is also susceptible to the Cu/CNTs nanocomposite. The main industrial disadvantage of Bacillus sp. in industrial settings is food spoilage.
which again can be controlled by Cu/CNTs. Therefore, to summarize, Cu/CNTs are a decent candidate for antimicrobial applications and are cheaper than other prevalent bactericidal nanoparticles. More work is needed in this direction with single walled CNTs used instead of multi-walled CNTs, and also in elucidating the mechanism of bactericidal action.

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