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Research Article

EVALUATION OF ANTIBACTERIAL EFFICACY OF CHEMICALLY SYNTHESIZED COPPER AND ZEROVALENT IRON NANOPARTICLES

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ABSTRACT

Objective: Growing resistance of microorganisms to potent antibiotics has renewed a great interest towards investigating bactericidal properties of nanoparticles and their nanocomposites as an alternative. Therefore, the present study has been carried out to investigate and compare the antibacterial properties of copper (Cu) and zerovalent iron (Fe^{o}) nanoparticles. **Methods:** Nanoparticles were synthesized by reducing aqueous solution of respective salt solution with sodium borohydride ($NaBH_4$). The synthesized particles were further characterized by X-Ray Diffractogram (XRD), and Scanning Electron Microscopy (SEM) techniques to analyze size and morphology respectively. Antibacterial efficacy of metal nanoparticles was evaluated by agar well diffusion method. **Results:** Average size of the particles was found to be 17.25 nm (Cu) and 44.87 nm (Fe^{o}). Energy-dispersive spectrum (EDS) of the nanoparticles dispersion confirmed the presence of elemental metals. The mechanism of microorganism inactivation is considered as species-dependent . *Pseudomonas aeruginosa* exhibited highest antibacterial sensitivity (26.00 ± 0.41 mm) to copper nanoparticles whereas *Bacillus cereus* offered maximum zone of inhibition (23.33 ± 0.89 mm) to Fe^{o} nanoparticles. **Conclusion:** Results from this study signify that the Cu and Fe^{o} nanoparticles potentiate the antibacterial action of both gram positive and gram negative bacteria.

Keywords: Nano copper, Zerovalent iron, Aqueous reduction method, Bactericidal effect, Bacterial pathogens, Zone of inhibition.

INTRODUCTION

Disease causing microbes that have become resistant to drug therapy are an increasing public health problem. One of the measures to combat this increasing rate of resistance is to have continuous investigations into new, safe and effective antimicrobials as alternative agents to substitute with less effective ones [1]. Owing to their high antibacterial properties, nanoparticles of silver, oxides of Zinc, titanium, copper, and iron are the most commonly used nanoparticles in antimicrobial studies. Furthermore, these nanoparticles have been used to deliver other antimicrobial drugs to the site of pathological process [2]. In general, a variety of preparation routes have been reported for the preparation of metallic nanoparticles. However, aqueous reduction method is most widely employed because of its advantages such as simple operation, high yield and quality, limited equipment requirements and ease of control. Because of strong reducing ability, $NaBH_4$ is widely used as a reductant for this aqueous reduction process [3]. Although metals and metal oxides are known to be toxic at relatively high concentrations, they are not expected to be toxic at low concentrations. Several resistance mechanism to metals have been described, the most common, which is enhanced efflux of metal ions from the cell, is a high-level, single-step and target-based mutation. This mutation enhances efflux of metal ions from cell and makes metal resistance less probable owing to its multifaceted mode of action [4]. Recent studies have demonstrated that specially formulated metal oxide nanoparticles have good antibacterial activity and antimicrobial formulations comprising nanoparticles could be effective bactericidal materials. Wei *et al.* [5] reported that the bactericidal effects of metal nanoparticles are impacted by the release of metal ions in solution. Typically, antimicrobials kill bacteria by binding to some vital compounds of bacterial metabolism, thereby inhibiting the synthesis of functional biomolecules or impeding normal cellular activities. Nanoparticles attached to the microbial surfaces can decrease both cell mobility and nutrient flow between the cell's exterior and interior compartments [6]. Kim *et al.* [7] investigated that the antimicrobial effect of Fe^{o} has been attributed to involve the generation of intracellular oxidants (e.g. HO^{o} and Fe^{IV}) produced by the reaction with hydrogen peroxide or other species as well as a direct interaction of Fe^{o} with cell membrane components. Raffi *et al.* [8] reported that copper has the potential to disrupt cell function in multiple ways, since several mechanisms acting simultaneously may reduce the ability of microorganisms to develop resistance against copper. The nonspecific mode of action of nanoparticles against bacteria makes them ideal candidates as antimicrobial agents with less risk of development of bacterial resistance [9]. Therefore, our aim in the present study was to synthesize copper and zerovalent iron nanoparticles and investigate the antibacterial effect against selected gram positive and gram negative bacteria.

MATERIALS AND METHODS

MATERIALS

Copper sulfate pentahydrate ($CuSO_4.5H_2O$), Sodium borohydride ($NaBH_4$), Sodium citrate ($Na_3C_6H_5O_7$), Ferrous sulfate heptahydrate ($FeSO_4.7H_2O$), Ethanol, Acetone, and standard antibiotic disc were purchased from Himedia (P) Ltd, Mumbai and used as starting materials without further purification. Milli-Q water was used for the fabrication of nanoparticles.

METHODS

Preparation of nanoparticles

Copper

The preparation of copper nanoparticles was followed the method according to the description of Samim *et al.* [10]. The particles were prepared in aqueous phase by chemical reduction of cupric salt solution using sodium borohydride in the presence of sodium citrate as a capping agent. In a typical set, 10 ml of 0.0125 M $CuSO_4$ solution was purged with nitrogen (N_2) gas for 10 min to remove the dissolved oxygen (DO) and 10 ml of 0.2 M sodium citrate solution was added and allowed to stir for 30 min under N_2 atmosphere. 1 ml of aqueous solution of $NaBH_4$ (0.1 M) was then added drop wise to it under constant stirring in N_2 atmosphere. The solution color

changed to dark brown on complete addition of reducing agent indicating the formation of citrate capped copper nanoparticles. The particles were then extracted by ultracentrifugation of resultant solution at 10,000 rpm for 1 h. The particles thus obtained were washed twice with acetone before being vacuum dried at 50°C for 12 h to remove water. The dried particles were used for further characterization.

Zerovalent iron

The preparation of Fe^{o} nanoparticles was followed the method according to He and Zhao [11] with slight modifications. In brief, the preparation was carried out in a 250 ml flask attached to a vacuum line. Before use, deionized (DI) water was purged with purified N_2 gas for 15 min to remove dissolved oxygen (DO). In a typical preparation, a stock solution of 0.21 M $FeSO_4.7H_2O$ was prepared right before use. Fe concentration used in this study was 0.1 g/L. The Fe^{2+} ions were then reduced to Fe^{o} by adding a stoichiometric amount of $NaBH_4$ aqueous solution at a BH_4^-/Fe^{2+} molar ratio of 2.0 to the mixture with magnetic stirring at 230 rpm under ambient temperature. The ferrous ion was reduced to zero-valent iron according to the following reaction:

$$Fe(H_2O)_6^{2+} + 2BH_4^- \rightarrow Fe^{o}\downarrow + 2B(OH)_3 + 7H_2\uparrow$$

The resultant black particles were separated from the solution by centrifugation at 4000 rpm for 5 min and washed with N_2 saturated deionized water and at least three times with 99% absolute ethanol. Finally, the synthesized Fe^{o} nanoparticles were dried in an oven at 60°C and used for further characterization.

Characterization of nanoparticles

Visual observation

The reduction of metal ions was roughly monitored by visually observing the change of color in the reaction solution.

X-ray diffractogram

XRD patterns of synthesized nanoparticles were recorded with an X'pert PROPAN analytical instrument operated at 40 kV and a current of 30 mA with Cu α radiation (λ=1.54060 Å). A continuous scan mode was used to collect 2θ data from 10.08° to 79.93°. The diffraction intensities were compared with the standard JCPDS files. Crystalline size of the nanoparticles was calculated from the line broadening of X-ray diffraction peak according to the Debye-Scherer formula

$$D = k\lambda / \beta \cos\theta,$$

Where D is the thickness of the nanocrystal, 'k' constant, 'λ' wavelength of X-rays, 'β' width at half maxima of reflection at Bragg's angle 2θ, 'θ' Bragg's angle.

Scanning electron microscopy

Size and morphology of the nanoparticles was examined by SEM (SU 1510) operated at 5 kV, magnification x10 k. Thin film of the sample was prepared on a carbon coated copper grid by just dropping the suspension of nanoparticles in water on the grid, extra solution was removed using blotting paper and then the film on the SEM grid were allowed to dry by putting it under a mercury lamp for 5 min. The sample surface images were taken at different magnifications.

Energy dispersive spectroscopy

EDS was used for the determination of elemental composition and purity of the samples by atom percentage of metal. Elemental analysis on nanoparticles was carried out using EDS instrument (JSM 35 CF JEOL) in a resolution of 60 Å, operated at 15.0 kV with a magnification of about 5 k. Samples were prepared on a carbon coated copper grids and kept under vacuum desiccation for 3 h before loading them onto a specimen holder.

Antibacterial studies

Bacterial culture

The following bacterial pathogens namely *Bacillus cereus, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella typhimurium, Staphylococcus aureus, Streptococcus epidermis,* and *Yersinia pestis* were procured from the Microbial Type Culture Collection (MTCC), Chandigarh, India. All the cultures were grown on nutrient agar plates and maintained in the nutrient agar slants at 4°C. Overnight culture in the nutrient broth was used for the present study.

Screening for antibacterial activity

The antibacterial activity of chemically fabricated Cu and Fe^{o} nanoparticles nanoparticles against the pathogenic bacteria was screened by agar well diffusion method. Pure bacterial culture was sub cultured in nutrient broth for 24 h at 37°C. Each strain was swabbed uniformly onto the individual Mueller Hinton agar (MHA) plates using sterile cotton swabs. Wells of 6 mm diameter were made on Mueller Hinton agar plates using sterile gel puncture. Using a micropipette, 50 µl of nanoparticles suspension was dispensed onto each well on all the plates. The plates were incubated at 37°C for 24 h. After incubation, the presence of bacterial growth inhibition zone around the sample loaded well was absorbed and their diameters (mm) were measured using measuring scale. Each nanoparticles was tested in triplicate with broad spectrum antibiotic gentamycin (10 mcg/disc) as standard.

Assessment of activity index

According to Singariya *et al.* [12], the assessment of activity index was obtained by comparing the resultant inhibition zones of nanoparticles with the standard reference antibiotic using the formula,

$$\text{Activity index (AI)} = \frac{\text{Inhibition zone of the sample}}{\text{Inhibition zone of the standard}}$$

Assessment of fold increase

Increase in fold area was assessed by calculating the mean surface area of the inhibition zone generated by the standard reference antibiotic and nanoparticles [13]. The fold increase area was calculated by the equation,

$$\text{Fold increase (\%)} = (b-a)/a \times 100$$

Where a and b refer to the inhibition zones of antibiotic and nanoparticles respectively.

RESULTS AND DISCUSSION

Characterization of nanoparticles

Visual inspection

The appearance of dark brown colloidal solution for Cu and black for Fe^{o} in the reaction mixture indicated the formation of respective nanoparticles (Figure 1). The formation of color in the reaction solution arises from excitation of surface Plasmon vibration in the

metal nanoparticles [14].

Figure 1: Formation of copper (left) and zerovalent iron (right) nanoparticles in the reaction mixture

X-ray diffractogram

The XRD pattern shows that the synthesized nanoparticles are in amorphous stage and in tetragonal system. The XRD pattern clearly showed the crystalline nature of nanoparticles (Figure 2). The diffraction peaks at 2θ value of 38.51°, and 44.80° correspond to the (111) and (110) reflections plane of copper and iron respectively.

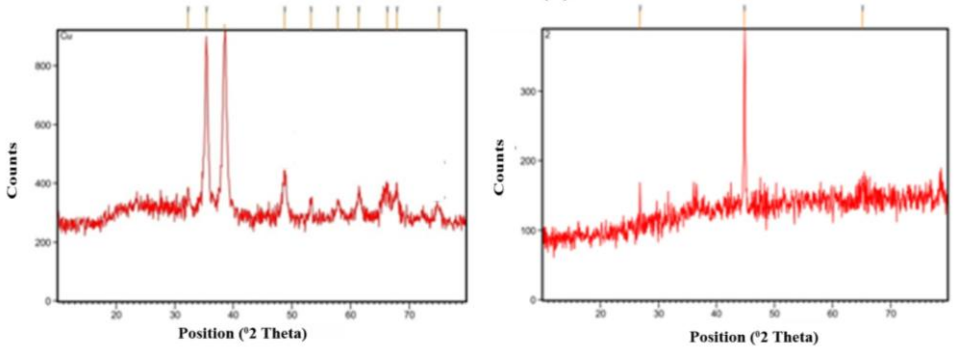

Figure 2: X-ray diffractogram of copper (left) and zerovalent iron (right) nanoparticles

The different peaks in XRD pattern are indexed and the corresponding values of interplanar spacing "d" are calculated and compared with standard JCPDS-ICDD, PDF, Files. The XRD study confirms that the resultant particles are face centered cubic (fcc) metal nanoparticles. The obviously broadened diffraction peaks suggest that the resultant nanoparticles should have a very small crystalline size and its size is found to be 17.85 nm (Cu), and 44.87 nm (Fe^{o}).

Scanning electron microscopy

Scanning electron micrograph of the synthesized nanoparticles is presented in Figure 3. The

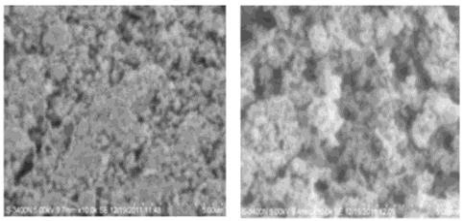

Figure 3: Scanning electron micrograh of copper (left) and zerovalent iron (right) nanoparticles

micrograph shows that the appearance of the particles is spherical in shape. Synthesized particles do not appear as discrete one but form much larger particles. The observations of such larger nanoparticles are composed of van der Waals clusters of smaller entities and magnetic interactions among the particles.

Energy dispersive spectroscopy

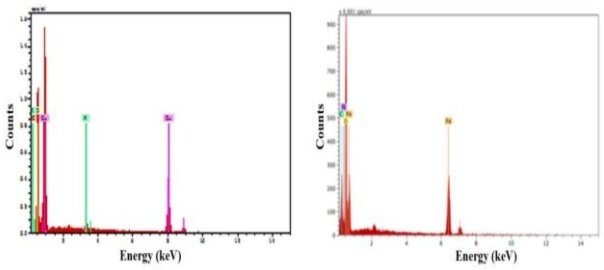

Figure 4: Energy dispersive spectrum of copper (left) and zerovalent iron (right) nanoparticles

EDS micrograph explains the surface atomic distribution and chemical composition of nanoparticles. Quantitative measuring results obtained from EDS analysis reflect that 97.07% and 72.11% atom particles were of copper and iron respectively which confirms the purity of copper and iron metals. The other weaker signals of K, C and O are owing to used precursor salts for the synthesis of nanoparticles (Figure 4).

Antibacterial effect

Antibacterial activity of chemically synthesized Cu and Fe^{o} nanoparticles was evaluated against pathogenic bacteria using standard zone of inhibition (ZOI) assay. The greatest inhibitory effect was observed against *Pseudomonas aeruginosa* with a zone of inhibition of 26.00 ± 0.41 mm for Cu nanoparticles and *Bacillus cereus* (23.33 ± 0.89 mm) for Fe^{o} nanoparticles (Table 1).

Table 1: Antibacterial activity of copper (Cu) and zerovalent iron (Fe^{o}) nanoparticles against gram positive and gram negative bacterial pathogens

Bacterial pathogens	Zone of inhibition (mm) (Mean ± S.D)	
	Cu	Fe^{o}
Bacillus cereus (G +ve)	19.00 ± 0.82	23.33 ± 0.94
Escherichia coli (G –ve)	14.00 ± 0.82	15.00 ± 0.37
Klebsiella pneumoniae (G –ve)	11.83 ± 0.62	19.10 ± 0.37
Pseudomonas aeruginosa (G –ve)	26.00 ± 0.41	11.33 ± 1.25
Salmonella typhimurium (G –ve)	15.10 ± 0.29	11.67 ± 0.94
Staphylococcus aureus (G +ve)	13.83 ± 0.62	12.00 ± 0.41
Streptococcus epidermis (G +ve)	14.97 ± 0.37	14.77 ± 0.21
Yersinia pestis (G –ve)	12.50 ± 1.08	11.63 ± 0.26

Note: G +ve – Gram Positive; G –ve – Gram Negative

The presence of an inhibition zone clearly indicates the mechanism of the biocidal action of nanoparticles involves disrupting the membrane. Extend of inhibition depends on the concentration of nanoparticles as well as on the initial bacterial concentration. The reason could be that the smaller size of the particles which leads to increased membrane permeability and cell destruction [15]. Because of the large surface area of the nanoparticles, it could be tightly adsorbed on the surface of the bacterial cells so as to disrupt the membrane, which would lead to the leakage of intracellular components, thus killing the bacterial cells [16]. Padmavathy and

Vijayaraghavan [17] reported that the size of the inhibition zone increased significantly with decreasing size of the nanoparticles. It is reasonable to state that binding of the nanoparticles to the bacteria depend on the surface available for interaction. Smaller particles having the larger surface area available for interaction will give more bactericidal effect than the larger particles [18]. Activity index (AI) for Cu and Fe^{o} nanoparticles is given in Table 2.

Table 2: Activity index (AI) for copper (Cu) and zerovalent iron (Fe^{o}) nanoparticles against gram positive and gram negative bacterial pathogens

Bacterial pathogens	Activity Index	
	Cu	Fe^{o}
Bacillus cereus (G +ve)	1.90	2.33
Escherichia coli (G –ve)	1.00	1.07
Klebsiella pnuemoniae (G –ve)	1.48	2.39
Pseudomonas aeruginosa (G –ve)	2.17	0.94
Salmonella typhimurium (G –ve)	1.37	1.06
Staphylococcus aureus (G +ve)	0.92	0.80
Streptococcus epidermis (G +ve)	0.83	0.82
Yersinia pestis (G –ve)	1.25	1.16

Note: G +ve – Gram Positive; G –ve – Gram Negative

It shows that all the tested bacteria exhibited excellent antibacterial activity than the standard antibiotic gentamycin. Among the tested strains, Fe^{o} nanoparticles demonstrated highest percentage fold increase (138.75%) against *K. pneumoniae* followed by CuNPs (116.67%) for *P. aeruginosa* (Table 3).

Table 3: Percentage fold increase of copper (Cu) and zerovalent iron (Fe^{o}) nanoparticles with reference to standard antibiotic gentamycin against gram positive and gram negative bacterial pathogens

Bacterial pathogens	Fold Increase (%)	
	Cu	Fe^{o}
Bacillus cereus (G +ve)	90.0	133.30
Escherichia coli (G –ve)	0.00	7.14
Klebsiella pnuemoniae (G –ve)	47.88	138.75
Pseudomonas aeruginosa (G –ve)	116.67	-5.58
Salmonella typhimurium (G –ve)	37.27	6.09
Staphylococcus aureus (G +ve)	-7.80	-20.00
Streptococcus epidermis (G +ve)	-16.83	-17.94
Yersinia pestis (G –ve)	25.00	16.30

Note: G +ve – Gram Positive; G –ve – Gram Negative

The detailed mechanism for the activity of metal nanoparticles is still under debate. One possible explanation of the antibacterial effect is that the ions released by the nanoparticles may attach to the negatively charged bacterial cell wall and rupture it, thereby leading to protein denaturation and cell death [19]. There is also potential for multiple adverse interactions such as oxidative stress and inflammatory responses [5]. Such cellular processes may lead to cell death via cell necrosis or apoptosis (programmed cell death).

CONCLUSIONS

Accompanying the rapid advance of nanotechnology, several metal and metal oxide nanoparticles have shown promise as strong antimicrobial agents against a broad spectrum of microorganisms. In this present investigation, nontoxic nanomaterials which can be prepared in a simple and cost effective manner have great promise as antibacterial agents and it exhibits the broad spectrum of antibacterial activity. Hence, the present study is useful towards authenticating the nanoparticles to be a potent antibacterial agent. Though this research is very preliminary, provides helpful insights to the development of novel antibacterial agents. Meanwhile, more elaborate experimental evidences are needed to elucidate the mechanism of antibacterial effect of Cu and Fe^{o} nanoparticles.

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