International Journal of Applied Pharmaceutics

ISSN- 0975-7058 Vol 10, Issue 6, 2018

Original Article

CHARACTERIZATION AND ANTIBACTERIAL ACTIVITY OF ZnO NANOPARTICLES SYNTHESIZED BY CO PRECIPITATION METHOD

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Received: 28 Aug 2018, Revised and Accepted: 21 Sep 2018

ABSTRACT

Objective: In the present study the antibacterial activity of zinc oxide (ZnO) nanoparticles was investigated against gram negative (*Escherichia coli* and *Proteus vulgaris)* and gram positive (*Staphylococcus aureus* and *Streptococcus mutans*) organisms.

Methods: The synthesis of ZnO nanoparticles was carried out by co-precipitation method using zinc sulfate and sodium hydroxide as precursors. These nanoparticles were characterized by XRD (X-Ray Diffraction), FTIR (Fourier Transform Infrared Radiation), UV-Visible spectroscopy and SEM (Scanning Electron Microscope) with EDX (Energy Dispersive X-ray analysis). As well as antibacterial activity and minimum inhibitory concentration of the nanoparticles were carried out by agar well diffusion method and broth dilution method respectively against gram negative (*Escherichia coli* and *Proteus vulgaris*) and gram positive (*Staphylococcus aureus* and *Streptococcus mutans*) bacteria.

Results: The average crystallite size of ZnO nanoparticles was found to be 35 nm by X-ray diffraction. The vibration bands at 450 and 603 cm^{-1} which were assigned for ZnO stretching vibration were observed in FTIR spectrum. The optical absorption band at 383 nm was obtained from UV-Visible spectrum. Spherical shape morphology was observed in SEM studies. The antibacterial assay clearly expressed that *E. coli* showed a maximum zone of inhibition (32±0.20 mm) followed by *Proteus vulgaris* (30±0.45 nm) at 50 mg/ml concentration of ZnO nanoparticles.

Conclusion: Zinc oxide nanoparticles have exhibited good antibacterial activity with gram negative bacteria when compared to gram positive bacteria.

Keywords: ZnO nanoparticles, XRD, FTIR, UV-VISIBLE spectroscopy, SEM, EDX, MIC

DOI: http://dx.doi.org/10.22159/ijap.2018v10i6.29376

INTRODUCTION

Development of antibiotic resistant strains has become a serious global problem now a day. One of the promising approaches for overcoming bacterial resistance towards the antibiotics is the use of metallic nanoparticles [1]. Recently, Inorganic antimicrobial agents are used for the control of organisms in various fields [2]. By bringing down the size of bulk material of inorganic metal oxides to nano, it alters their activity and enhances physical, chemical and biological properties [3]. It has been illustrated that highly reactive metal oxide nanoparticles exhibit striking biocidal action against gram positive and gram negative bacteria [4]. Sawai [5] projected that production of hydrogen peroxide be a main factor for the antibacterial function, while Stoimenov [4] elucidated that the binding of ZnO nanoparticles on the bacterial surface due to electrostatic forces might be a mechanism. The mechanism of action of metal oxide nanoparticles on microorganism alters cell membrane properties, i.e., mainly permeability that facilitates particle penetration inside the microorganism, leading to cellular DNA damage [6]. Generation of reactive oxygen species (ROS) through photocatalysis might be responsible for the biological function of zinc oxide and titanium oxide nanoparticles [7].

The biological activity of the zinc oxide nanoparticles mainly depends on its size, surface area and concentration of the nanoparticle. The antibacterial activity of Zinc oxide nanoparticles along with antibiotics was studied against gram positive (*Bacillus subtilis)* and gram negative (*E. coli*) bacteria using disc diffusion method byRavichandrika *et al.* [8]. Results indicated that zinc oxide nanoparticles enhance the bactericidal activity of macrolides, tetracyclines and beta-lactam antibiotics and similar findings were observed byManyasree and Kiranmayi [9].

Table 1 shows the method and conditions of synthesis plays a key role in determining the size of the nanomaterial. There are few methods such as co-precipitation [10, 11] sol-gel processing [12] micro-emulsions processing, hydrothermal solvo-thermal processing [13], microwave processing, sono-chemical processing and template processing, high temperature solid state reaction, high energy ball milling [14-16] liquid mix process [17], rapid quenching process [18], thermal plasma [19], UV irradiation and lithography are used for synthesizing nanoparticles.

Table 1: Synthesis of nanoparticles by using various methods

Method of synthesis	Synthesis conditions	Size of nanomaterial(nm)	Reference
Mechanochemical	300-450 °C	51	[20]
Precipitation	Calcination 2h at 600 °C	50	[21]
Precipitation in presence of surfactants	Calcination 2h at 500 °C	54-60	[22]
Sol-gel method	Calcination at 500 °C, drying 24h at 80 °C	100	[23]
Microwave techniques	100-200 °Creaction 5-10h	55-110	[24]
Micro emulsion	15h, 140 °C,drying 60 °C	45	[25]

In the present research work, an attempt has been made to synthesize the ZnO nanoparticles by co-precipitation method and investigate the antibacterial activity and minimum inhibitory concentration of ZnO nanoparticles against gram negative and gram positive bacteria.

MATERIALS AND METHODS

Zinc sulphate and Sodium hydroxide, involved in the synthesis of ZnO nanoparticles were purchased from Merck chemicals. The test organisms, *E. coli* (MCC 2412) and *Staphylococcus aureus* (MCC-

2408) were procured from MCC, Pune, India. *Proteus vulgaris* (MTCC-426) and *Streptococcus mutans* (MTCC-497) were collected from MTCC, Chandigarh, India. Media required for the cultivation of microorganisms are Nutrient agar (*E. coli* and *Proteus vulgaris*)Trypticase soy yeast extract agar (*Staphylococcus aureus*) and Brain heart infusion agar (*Streptococcus mutans*) were obtained from Hi-Media Pvt Ltd. All the chemicals used in this experiment were analytical grade and used without further purification.

Synthesis of ZnO nanoparticles

The zinc oxide nanoparticles were prepared by co-precipitation method. 1M of zinc sulfate was dissolved in distilled water and the solution was kept under constant stirring using magnetic stirrer for one hour. After complete dissolution of zinc sulfate, 2M sodium hydroxide solution was added under constant stirring, drop by drop touching the walls of the vessel. The reaction was allowed to proceed for 2 h. At the end of the reaction, the white creamy solution was formed and was allowed to settle for overnight. The precipitate was washed several times using distilled water then dried at 80 °C. Obtained product was kept at 700 °C for 3h in a muffle furnace. During drying, complete conversion of zinc hydroxide into zinc oxide takes place [26].

Characterization of ZnO nanoparticles

The crystal structure of the sample was analyzed by means of XRD-6100 diffractometer (Shimadzu), and the patterns were recorded with Copper Kα radiation (λ=1.54060 A °). Molecular analysis of the samples was performed by Fourier transform infrared (FT-IR) spectroscopy using IR Affinity-1S (Shimadzu) spectrometer, recorded in the wave number range of 400–4,000 cm^{-1}. The maximum optical absorption of the sample was characterized by UV-Visible Spectroscopy (JASCO V 670), in the range of 200-800 nm. Morphological study of the nanoparticles was carried out with scanning electron microscope (SEM) (EVO 18 carlzeiss).

Agar well diffusion method

The antibacterial activity of the nanoparticles was determined by agar well diffusion method [27] against both gram negative and gram positive microorganisms. Once the medium was solidified, a suspension of each sample of the bacteria was diluted prior to 10^{-1}, 10^{-2} and 10^{-3}(1 ml of 108 cells/ml) and was spread on a solid agar medium in petri plates. The wells were prepared by using sterile cork borer (6 mm). Each well was filled with different concentrations of nanomaterial ranging from 10-50 mg/ml. The plates were incubated at 37 °C for 24 h. The zone of inhibition was measured with mean±SD values.

Minimum inhibitory concentration

Broth dilution method [28] was used to determine the minimum inhibitory concentration of ZnO nanoparticles. A series of 4 test tubes were taken add 10 ml of media and a loop full of culture to all the test tubes and finally add 2 mg/ml, 4 mg/ml, 6 mg/ml and 8 mg/ml of nanoparticle suspension to each test tube. The test tube without bacterial suspension is considered as control. Keep the test tubes for overnight incubation at 37 °C temperature. Read the absorbance at 600 nm using spectrophotometer. MIC is where the absorbance value of sample equals to or near to control [29].

RESULTS AND DISCUSSION

Co-precipitation method

The zinc oxide nanoparticles were prepared by co-precipitation method. The synthesis technique is a good choice among all because of simple, direct and rapid procedure [30], homogeneous mixing of reactants [31], easy control of particle size [32], shape and composition of the nanomaterial [33].

Powder X-ray diffraction (XRD) studies

Powder XRD is a rapid analytical technique primarily used for phase identification of a crystallite material and can provide information on unit cell dimensions. XRD pattern of the synthesized ZnO nanopowder is in hexagonal phase. The sharper and stronger diffraction peaks were observed from fig.1. The diffraction peaks at 31.75 °, 34.40 °, 36.23 °, 47.53 °, 56.59 °, 62.85 °, 66.39 °, 67.94 ° and 69.07 ° 2θ which are associated with (100), (002), (101), (102), (110), (103), (200), (112) and (201) planes, respectively and were indicated in table 2. The (hkl) values are well agreed with the standard cards of ZnO (JCPDS file No: 79-2205). The average crystallite size of the sample (D) is calculated using Debye Scherrer's formula:

$$D = 0.9\lambda/\beta\cos\theta$$

Where, λ is the wavelength of the X-ray radiation, θ is the diffraction angle and β is the full width half maximum (FWHM) intensity. On substituting the values λ = 1.5408, β = 0.041, cosθ = 0.9504 in Debye Scherrer's formula (0.9×1.5408) ÷ (0.041×0.9504) = 35 nm. The prominent peaks were used to estimate the grain size of sample with the help of Scherrer's equation and it can be indexed to the hexagonal crystal structure. The calculated average crystallite size is found to be 35 nm. Crystallite size of the nanoparticles was determined from the line broadening of X-ray diffraction peak by using above mentioned formula. The evaluated cell parameters a=0.32511 and c =0.52069 nm are in close agreement with the reported values.

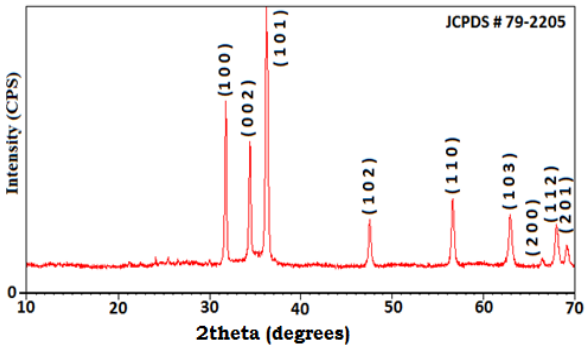


Fig. 1: XRD patterns of the zinc oxide nanoparticles

Table 2: The observed and standard 2θ values of XRD data of zinc oxide nanoparticles

Observed 2θ	Standard 2θ	h k l
31.75	31.76	1 0 0
34.40	34.41	0 0 2
36.23	36.25	1 0 1
47.53	47.53	1 0 2
56.59	56.59	1 1 0
62.85	62.85	1 0 3
66.39	66.37	2 0 0
67.94	67.94	1 1 2
69.07	69.08	2 0 1

Fourier transform infrared spectroscopy (FTIR) analysis

Synthesized zinc oxide nanoparticles were subjected to FTIR spectral study to identify various functional groups related to the prepared nanoparticle. FTIR spectrum of synthesized zinc oxide nanoparticles was shown in fig. 2. The Peaks at 450 [34] and 603 are assigned to the Zn-O stretching vibration [8]. The peak at 1121 indicates the triply degenerative vibrational mode of sulphate ion.

The peaks at 1638 and 3405 are assigned to the bending and stretching vibration mode of water molecule.

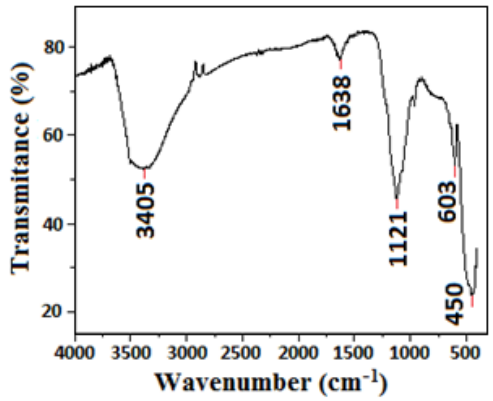


Fig. 2: FT-IR spectrum of zinc oxide nanoparticles

UV-Visible spectroscopy studies

UV-Visible spectroscopy is most widely used technique to investigate the optical properties of the particles. UV-Visible spectroscopy analysis was done in the range of 200-800 nm. Zinc oxide nanoparticles typically exhibit optical absorption around 370 nm [35-36]. The optical absorbance spectra of zinc oxide nanopowder showed in fig. 3. The band was observed at 383 nm assigned to the absorption of zinc oxide nanoparticles.

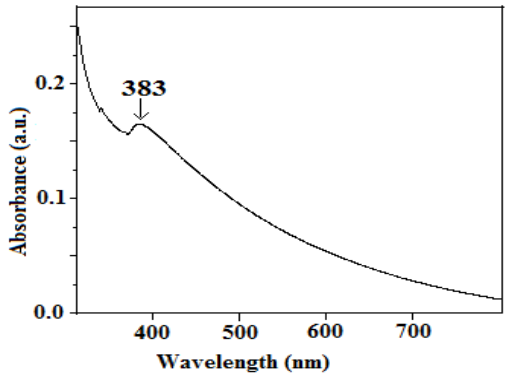


Fig. 3: Optical absorption spectrum of zinc oxide nanoparticles

Scanning electron microscope and EDX analysis

Fig. 4 represents the SEM images of zinc oxide nanopowder at different magnifications. Surface morphology of zinc oxide nanopowder was deduced by using scanning electron microscopy (SEM). The results revealed that particles in the sample were compactly arranged and shown spherical morphology [37]. The appearance of larger nanoparticles is due to van der Waals clusters of smaller entities and magnetic interactions among the particles. The purity of the product was further confirmed by EDX analysis. In fig.5 EDX elucidates the surface atomic distribution and chemical composition of nanoparticles. The quantitative analysis of EDX spectrum reflects the presence of Zn and O peaks clearly indicated that the product was of pure ZnO devoid of impurities. The other weaker signals of K, L and S are owing to use precursor salts for the synthesis of nanoparticles.

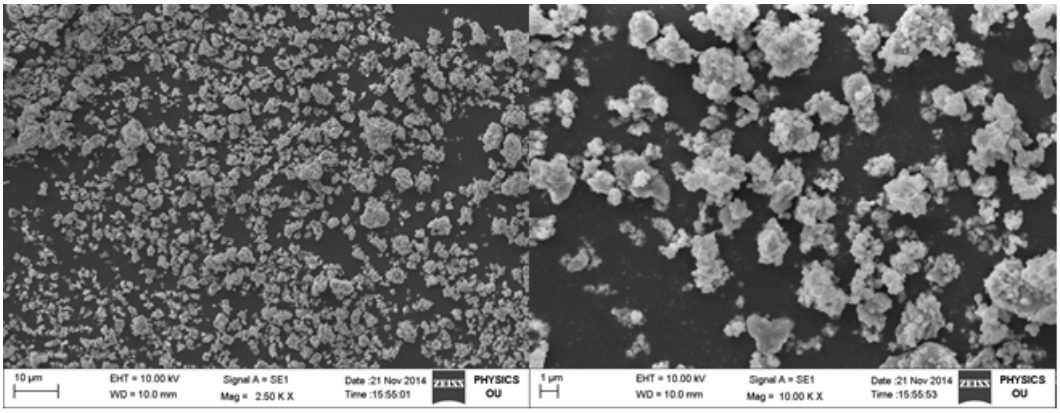
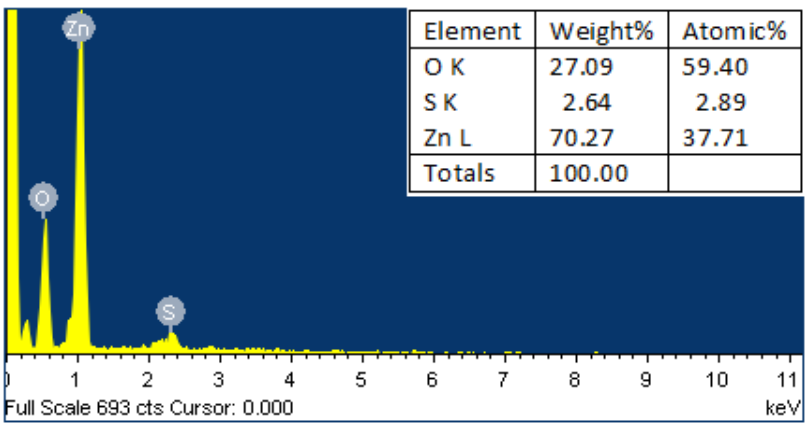


Fig. 4: SEM analysis of zinc oxide nanoparticles



Element	Weight%	Atomic%
O K	27.09	59.40
S K	2.64	2.89
Zn L	70.27	37.71
Totals	100.00	

Fig. 5: EDX spectrum of zinc oxide nanoparticles

Antibacterial activity of the zinc oxide nanoparticles

The antibacterial activity of zinc oxide nanoparticles was tested against gram negative bacteria *E. col i*(MCC-2412), *P. vulgaris* (MTCC-426) and gram positive bacteria *S. aureus* (MCC-2408) and *S. mutans* (MTCC-497) by agar well diffusion method. In fig.6 antibacterial activities of zinc oxide nano particles at different concentrations 10 mg/ml, 20 mg/ml, 30 mg/ml, 40 mg/ml, 50 mg/ml against both gram negative and gram positive bacteria were shown. The diameter of inhibition zones around each well is measured in millimeters and represented in table 3. Similar results were published by Rizwan *et al*., 2010 [38]. Results have indicating that the degree of zone of inhibition (with mean±SD values) was more against gram negative bacterial strains *E. coli* (32±0.20 mm) and *Proteus vulgaris* (30±0.45 mm) when compared to the gram positive bacteria (*Staphylococcus aureus*with24±0.35 mm *and Streptococcus mutans*with23±0.30 mm). The formation of an inhibition zone evidently states the mechanism of the biocidal action of nanoparticles involves disrupting the membrane. Range of inhibition depends on the concentration of nanoparticles as well as on the initial bacterial concentration. Due to the smaller size of the nanoparticles they can easily pass through the membrane and can destruct the cell. The important feature of the nanoparticles is large surface area, so that they can tightly bind to the surface of the bacterial cells to disrupt the membrane which would lead to the leakage of intracellular components and that kills the bacterial cells. Nanotoxicity may be attributed to electrostatic interaction between nanoparticles with bacterial cell membrane. Generation of reactive oxygen species (ROS) like hydroxyl ions, peroxides, super oxides and hydrogen peroxide may be the main reason for nanotoxicity of zinc oxide nanoparticles. The lethal agent hydrogen peroxide which is actually produced from hydroxyl ions and peroxide free radicle could damage the cell by complete destruction of membrane of bacterial cells [39]. As well as differences in structural organization between both gram classes of bacterial cell wall i.e. due to the presence of thicker peptidoglycan layer in gram positive bacteria, they are less prone to nanotoxicity of ZnO nanoparticles when compared to gram negative bacteria. This is might be the reason for the obtained results that indicates high degree of inhibition zone in the case of gram negative bacteria compared to gram positive bacteria.

Table 3: Antibacterial activity of ZnO nanoparticles by agar well diffusion method

Organism	Zone of inhibition (mm)				
	10mg/ml	20mg/ml	30mg/ml	40mg/ml	50mg/ml
E. coli	7±0.25	14±0.30	21±0.25	28±0.30	32±0.20
P. vulgaris	6±0.25	12±0.35	18±0.45	24±0.25	30±0.45
S. aureus	6±0.35	10±0.15	14±0.10	19±0.15	24±0.35
S. mutans	4±0.25	9±0.35	13±0.35	18±0.35	23±0.30

Number of experiments n=2, mean±SD

Minimum inhibitory concentration

In broth medium, MIC is where the absorbance value of sample equals to or near to control [29]. In solid medium, lowest concentration of nanoparticle suspension at which the bacterial colonies appeared on the top of the fresh medium in petri dishes is the MIC [40]. Minimum inhibitory concentration (MIC) of the zinc oxide nanopowder was determined by using broth dilution method. MIC was determined at different concentrations like 2 mg/ml, 4 mg/ml, 6 mg/ml and 8 mg/ml and control against two gram positive and two gram negative organisms.

The results showed significant MIC values between 2 mg/ml to 8 mg/ml concentration. *E. coli and Proteus vulgaris* showed MIC at 6 mg/ml*, streptococcusmutans* showed MIC at 8 mg/ml *and staphylococcusaureus* showed MIC at 4 mg/ml for zinc oxide nanopowder in table 4.

Table 4: Minimum inhibitory concentration (MIC) values in (mg/ml) of ZnOnano powder

Bacterual strain	MIC(mg/ml)
E. coli	6
P. vulgaris	6
S. aureus	4
S. mutans	8

CONCLUSION

Based on the results, it can be concluded that gram negative organisms have exhibited more sensitivity when compared to gram positive organisms to metal oxide nanoparticles [41]. The difference in the activity of both gram negative and gram positive bacteria might be due to structural and compositional variations of the cell membrane [42, 43, 44]. Gram positive bacteria have thicker peptidoglycan layer when compared to gram negative bacteria due to this kind of difference in structure, it is tough for nanoparticles to penetrate into membrane resulting in a low bacterial action [45]. All microbial species and strains are not exhibiting the same sensitivity to metal oxide nanoparticles [27]. The concentration of the nanoparticle plays a significant role in the resolution of antibacterial activity. The surface area of the metal oxide nanoparticles that comes in contact with bacterial cells is directly proportional to the extent of antimicrobial activity recommended by the particle.

AUTHORS CONTRIBUTIONS

All the author have contributed equally

CONFLICT OF INTERESTS

Declared none

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