Scientific paper

Preparation, Characterization and Antibacterial Activity of ZnO Nanoparticles on Broad Spectrum of Microorganisms

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Abstract

Nano particles have received increased attention regarding their potential utility in biomedicine. In this study, we have investigated the antibacterial activity of ZnO nano particles with various particle sizes. ZnO nano particles were synthesized by conventional precipitation method using zinc sulphate and sodium hydroxide as precursors followed by the calcinations of precipitates at 350 °C for 6 h (sample A) and 550 °C for 2 h (sample B). The products were characterized by X-ray diffraction (XRD) analysis and morphology of the particles was evaluated by Scanning Electron Microscopy (SEM). Antibacterial activities against four different microorganisms were evaluated by determining the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and zones of inhibitions using different concentrations of ZnO nanoparticles. The antibacterial activity was directly proportional to the concentration and inversely proportional to the particle size in all the microorganisms; moreover Gram positive bacteria were generally more affected than Gram negative bacteria. The stability of ZnO nanoparticles combined with potent antibacterial properties favours their application as antibacterials against broad spectrum of microorganisms.

Keywords: Zinc oxide nanoparticles, minimum inhibitory concentration, minimum bactericidal concentration, X-ray diffraction analysis

1. Introduction

In recent years, the unique and fascinating properties of nano structured materials have triggered tremendous motivation among scientists to explore the possibilities of using them in industrial and biotechnological applications. Inorganic materials such as metal and metal oxides have attracted lots of attention over the past decade due to their ability to withstand harsh process conditions.[1,2] Of the inorganic materials, metal oxides such as TiO_2, ZnO, MgO and CaO are of particular interest as they are not only stable under harsh process conditions but also generally regarded as safe materials to human beings and animals.[1,3] Zinc Oxide (ZnO), a wide band gap (3.36 eV) *II–VI* compound semiconductor, has attracted intensive research effort for its unique properties and versatile applications in transparent electronics, ultraviolet (UV) light emitters, piezoelectric devices, chemical sensors and spin electronics.[4–6] Zinc oxide is an interesting semiconductor material due to its application on solar cells, gas sensors, ceramics, catalysts, cosmetics and varistors. Invisible thin film transistors (TFTs) using ZnO as an active channel have achieved much higher field effect mobility than amorphous silicon TFTs.[7,8]

Emerging infectious diseases and the increase in the incidence of drug resistance among pathogenic bacteria have made the search for new antibacterials inevitable.[9] As the biological process occurs at nano scale and due to their amenability to biological functionalization the nanoparticles are finding important application in the field of medicine.[10] Potent antibacterial efficacy of nanoparticles is due to the large surface area to volume ratio and it has provided them an edge over their chemical counter parts which are finding the problem of drug resistance. Zinc

oxide has received increasing attention as antibacterial agent in recent years among the various metal oxides studied for their antibacterial activity, as zinc oxide nanoparticles are highly toxic to prokaryotic cells. Several reports have addressed the harmful impact of nanomaterials on living cells, but relatively low concentrations of ZnO are nontoxic to eukaryotic cells.[11] Moreover their stability under harsh processing conditions and relatively low toxicity combined with the potent antibacterial properties favour their application as antibacterials.[12] Antibiotics are the most common drugs to inhibit the growth and propagation of bacteria. However the use of antibiotics has various side effects.[13] The exploitations of novel substitutes to antibiotics, especially inorganic nanoparticles, have attracted more attention. There are numerous studies regarding the antibacterial effects of ZnO.[14–17] Most of these pertain to the antibacterial effect of bulk ZnO with larger particle size[18] and focus has been on *Escherichia coli* only. Little is known regarding interaction of nanoparticles with other bacteria and importantly less is known about the mechanism underlying the antibacterial effects.

We synthesized ZnO nanoparticles of varying sizes and tested their antibacterial activity against four different Gram positive and Gram negative bacteria. Various chemical synthesis methods have been employed by several workers to synthesize nano crystals such as solvothermal, hydrothermal, self assembly and solgel etc.[19,20] Spanhel and Anderson have explained the synthesis of nano crystals of zinc oxide using distillation set-up starting with products of Zinc acetate and ethanol.[21] In this work, the precipitation method was used. The materials obtained were thermally treated at various temperatures. The influence of temperature on structural, textural, and morphological properties of the materials were studied by powder X-ray diffraction (XRD) and Scanning Electron Microscopy (SEM).

2. Materials and Methods:

2. 1. Synthesis of ZnO Nanoparticles

There are different methods for preparing ZnO nanoparticles and the choice of the method depends on the final application. In this study, ZnO nano particles were prepared by conventional precipitation method with sodium hydroxide as precipitating agent. In this method, NaOH was added drop wise continually to aqueous zinc sulphate solution, at room temperature, in the molar ratio of 1:2 under continuous stirring. The resulting slurry was continuously stirred for 24 h and precipitate obtained was filtered, washed thoroughly with distilled water. The washing procedure was repeated several times and then collected residue was dried in an oven at 100 °C for 12 h, grinded to fine powder.[22] This powder was then divided into two parts and calcined at two different temperatures, sample A was calcined at 350 °C for 6 h and sample B was calcined at 550 °C for 2 h.

2. 2. Characterization of ZnO Nanoparticles

ZnO nanoparticles were characterized by two different methods; the compositional analysis of ZnO nanoparticles was done using X-ray diffraction (Panalytical X'Pert Pro) and the morphology of the synthesized powders was observed by SEM (Jeol, 5910LV).

2. 3. Preparation of ZnO Suspension for Antibacterial Activity

ZnO nano particle were prepared in the lab by the method described by Raju *et al.,* 2010.[22] ZnO nanoparticles were initially sterilized at 160 °C for 3 h, and then dispersed in ultrapure water (Milli-Q®, Millipore Corporation, Bedford, MA), vigorously vortexed for 10 min and additionally sonicated for 30 min to avoid aggregation and deposition of particles. The resulting suspensions (100 ml with concentration of 1 M) were considered as stock solution to be diluted and used for bacterial susceptibility evaluation.

2. 4. Bacterial Strains

Four bacterial strains including both Gram-positive [*Bacillus subtilis* (PCSIR-B-248), *Staphylococcus aureus* (ATCC-6538), *Streptococcus pyogenes* (ATCC-19615)] and Gram negative [*Escherichia coli* (PCSIR-B-67)]were obtained from PCSIR laboratories complex, Lahore, Pakistan. The cell suspensions used for antibacterial activity contained 10^5 colony forming units (CFU) ml^{-1}.

2. 5. Agar Diffusion Assay

Antibacterial tests were carried out by the agar diffusion method.[23] The bacteria for each experiment were freshly prepared by inoculating the nutrient broth and incubating at 37 °C for 24 h. Broth culture (0.6 ml) of test organisms added to 60 ml of molten agar with the help of sterile pipette, mixed well and poured into sterile petridish. Holes of 6 mm diameter were cut using sterile agar borer after hardening of agar. The 100 µl of test solution (ZnO of various concentrations) were poured into the holes using 0.1 ml pipette. The zones of inhibition were measured after 18 hours of incubation. Each experiment was made in triplicate and the inhibition zones are given as the mean ± standard deviation.

2. 6. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC and MBC were determined by a method recommended in NCCLS; 2000[24] with some modifications. Briefly, the sterile tubes were incubated aerobically at 37 °C for 24 h, which contained 5 ml Muller- Hinton (MH) broth (Difco, USA) with approximate 5×10^9 CFU

bacterial cells, zero zinc oxide nanoparticles (the control group) and various concentrations of zinc oxide nanoparticles. The concentration of tube without visible growth of the bacterial cells was the MIC. To evaluate the MBC, 100 µl of sample from each tube without visible growth was transferred into MH agar plate (Difco, USA), and then incubated aerobically for another 24 h. The concentration of the tube without growth was the MBC (in this test, the population in agar plate less than 10 was regarded no growth). All the measures were triplicate.

3. Results and Discussion

3. 1. Analysis Using XRD

The XRD patterns for sample A and B are shown in Fig. 1a and 1b respectively. The XRD patterns of both samples are consistent with the spectrum of ZnO, and no typical peaks attributable to possible impurities are observed. All major peaks correspond to hexagonal wurtzite crystal structure with (101) as the highest intensity peak. Intense and narrow peaks, observed in the XRD imply good crystalline nature of the synthesized ZnO nanoparticles. In addition, the broadening at the bottom of diffraction peaks also denotes that the crystalline sizes are small.[22] The crystallite size of both the samples was estimated by using Scherrer equation.

$$D = \frac{0.9\lambda}{\beta\cos\theta} \quad (1)$$

Specific surface area (SSA) is a material property. It is a derived scientific value that can be used to determine the type and properties of a material. It has a particular importance in case of adsorption, heterogeneous catalysis and reactions on surfaces. SSA can be calculated by using the equation.

$$S = \frac{6\times10^3}{D_p\rho} \quad (2)$$

Where S is the specific surface area, D_p is the size of the particles, and ρ is the density of ZnO (5.61 g/cm^3).[25] The calculated details of particle size and specific surface areas are reported in Table 1.

Comparison of the crystallite sizes of ZnO obtained in both samples indicates that sample B has a higher range of crystallite size than sample A, reflecting the effect of calcinations temperature and time on crystallite size.[26]

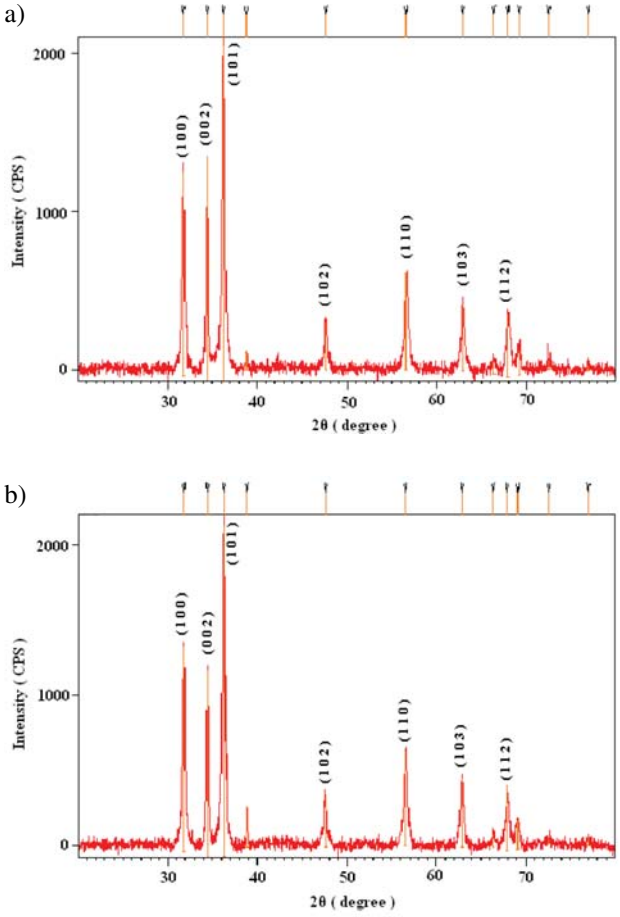

Figure 1. XRD pattern of ZnO nanoparticles; a) calcined at 350 °C for 6 hours b) calcined at 550 °C for 2 hours.

Table 1. The grain size and specific surface area of ZnO nanoparticles.

Sample A*				Sample B**			
2 θ (deg) of the Intense peak	FWHM# Intense peak (β) radians	Size of the particle (D) nm	specific surface area (m^2/g)	2 θ (deg) of the Intense peak	FWHM# Intense peak (β) radians	Size of the particle (D) nm	specific surface area (m^2/g)
31.7774	0.1722	46	23.4	31.8054	0.1722	46	23.4
34.429	0.123	65	16.5	34.4311	0.1476	54	19.8
36.2642	0.246	32	33.4	36.2538	0.1722	46	23.4
47.5626	0.2952	28	38.2	47.6074	0.0984	84	12.7
56.5696	0.123	70	15.3	56.5854	0.123	70	15.3
62.8342	0.246	36	29.7	62.8434	0.123	72	14.9
67.9684	0.2952	31	34.5	67.9137	0.0984	93	11.5

*ZnO nanoparticles calcined at 350 °C for 6 hours; ** ZnO nanoparticles calcined at 550 °C for 2 hours; # FWHM; Full width of half maximum

3. 2. Analysis Using SEM

Results of surface morphological and nano structural studies using SEM are summarized in Fig. 2a and 2b. Micrographs indicate that mono-dispersive and highly crystalline ZnO nanoparticles are obtained in both routes. The appearance is spherical in shape. The observation of some larger nanoparticles may be attributed to the fact that ZnO nanoparticles have the tendency to agglomerate due to their high surface energy and high surface tension of the ultrafine nanoparticles. According to Figs. 2a and 2b the increasing calcination temperature leads to the trend of increasing grain size. The fine particle size results in a large surface area that in turn, enhances the nanoparticles catalytic activity.

3. 3. Antibacterial Activity

There have been extensive studies to evaluate the antibacterial effect of ZnO and other oxide nanoparticles in vivo using mostly *E. coli*. In the present study, our interest was to determine if ZnO nanoparticles could be employed to inhibit the growth or kill several other diverse bacterial strains. The bacteriological tests were conducted with four strains as representatives of different bacteria types. *E. coli* was used as example for Gram-negative bacteria, *S. pyogenes* and *S. aureus* were used as a representative for Gram-positive bacteria, and *B. subtilis* was used as an example of endospores.

Agar disc diffusion method was used for the assessment of antibacterial activity. ZnO nanoparticles were dispersed in autoclaved Millipore water by ultrasonication. Aqueous dispersions of different desired concentrations were made. Antibacterial activity of ZnO nanoparticles against different bacteria is shown on basis of the inhibition zone size (mm) in Table 2. Here the zone of inhibition for both samples of ZnO nanoparticles is comparable with antibiotics like gentamicin and methicillin.

ZnO nanoparticles showed significant growth inhibition in a size dependent manner (Table 2). *B. subtilis*

a) b)

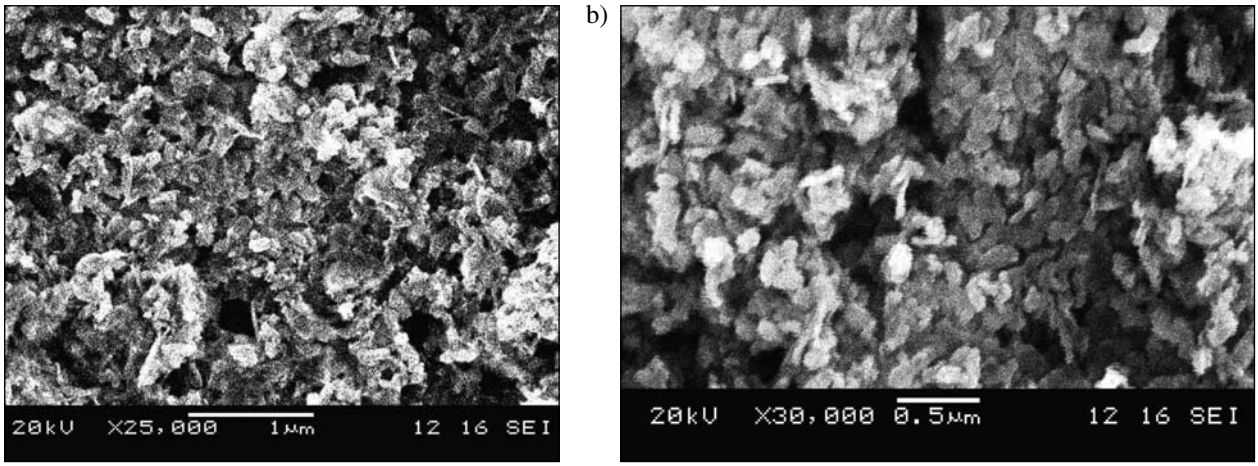

Figure 2. SEM micrographs of ZnO nanoparticles; a) calcined at 350 °C for 6 hours, b) calcined at 550 °C for 2 hours.

Table 2. Zones of inhibitions of ZnO nanoparticles against different bacterial strains.

Bioactive Agent		Zone of Inhibition* (Diameter, mm)			
		Escherichia Coli	***Bacillus subtilis***	***Staphylococcus aureus***	***Streptococcus pyogenes***
ZnO A**	100 µl	12 ± 1.2	16 ± 1.3	15 ± 0.5	10 ± 0.7
	300 µl	15 ± 0.8	25 ± 0.4	27 ± 1.6	13 ± 1.4
	500 µl	18 ± 0.5	32 ± 1.1	30 ± 0.4	19 ± 0.9
ZnO B***	100 µl	10 ± 0.6	12 ± 1.2	12 ± 0.9	9 ± 1.1
	300 µl	11 ± 0.4	15 ± 0.8	14 ± 1.4	11 ± 1.3
	500 µl	13 ± 0.9	18 ± 0.7	17 ± 0.4	14 ± 0.8
Methicillin	100 µl	20 ± 0.3	28 ± 0.4	34 ± 0.3	25 ± 0.6
Gentamycin	100 µl	18 ± 0.5	29 ± 0.6	32 ± 0.4	22 ± 0.7

* Inhibition zone (mm) include the diffusion assay disc diameter (6mm), which carried 100 µl from ZnO suspension. The diameter of inhibition zones are means triplicate ± standard deviation. $p < 0.05$ when compared with negative control i.e. blank/solvent ($p < 0.05$ is taken as significant). ** ZnO nanoparticles calcined at 350 °C for 6 hours. *** ZnO nanoparticles calcined at 550 °C for 2 hours

showed highest (32 mm) and *E. coli* showed lowest (18 mm) zone of inhibition at a dilution of 500 µl. In this work, we present a detailed and systematic study to understand the effect of particle sizes on antibacterial activity. To determine the antibacterial activity of the synthesized ZnO nanoparticles, we performed both liquid broth and plate based growth studies. Antibacterial activity of the ZnO nanoparticles was dose dependent. The MIC and MBC represents the antibacterial activity of the ZnO dispersed in batch cultures. The findings are summarized in Table 3.

Table 3. Minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations of ZnO* nanoparticles against different bacterial strains

Bacterial Strain	MIC (mM) ZnO nanoparticles*		MBC (mM) ZnO nanoparticles*	
	A**	B***	A**	B***
Escherichia Coli	21	32	43	55
Bacillus subtilis	10	18	28	37
Staphylococcus aureus	8	15	23	29
Streptococcus pyogenes	16	23	38	43

*1mM of ZnO nanoparticles = 72µg/ml
**ZnO nanoparticles calcined at 350 °C for 6 hours
*** ZnO nanoparticles calcined at 550 °C for 2 hours

The MIC was generally observed to be in the range of 8 to 32 mM (depending on the particular bacterial strain). In addition to further verify, the viable bacterial cells were determined by plating cultures from the growing cells in the presence of different concentrations of different sized ZnO nanoparticles. The MIC and MBC values of ZnO nanoparticles are observed to decrease with increase in ZnO concentration as well as decrease in crystallite size for all the four microorganisms. However, the *S. aureus* showed a higher response to the ZnO nanoparticles as compared to that of *E. coli*. The antibacterial activity of ZnO sample A was much stronger than that of ZnO sample B. This could be simply explained as smaller particles normally have a larger surface to volume ratio which provides a more efficient mean for antibacterial activity.[27] The generation of hydrogen peroxide (H_2O_2) presents another elucidation of the antibacterial activity of ZnO.[28] H_2O_2 that generates from the surface of ZnO is considered as an effective mean for the inhibition of bacterial growth.[29] It can be assumed that the concentration of H_2O_2 generated from the surface increases with decreasing particle size, because the number of ZnO powder particles per unit volume of powder slurry increases with particle size decreasing. Another possible mechanism for ZnO antibacterial activity is the release of Zn^{2+} ions. It is well known that ZnO normally becomes unstable in the solution, and when H_2O_2 is produced, the Zn^{2+} ion concentration is increased as a result of ZnO decomposition.[30] The obtained data, regarding the bacterial sensitivity to ZnO, indicated that Gram positive bacteria were more sensitive than Gram negative bacteria to ZnO nanoparticles this could be explained as the antibacterial action of ZnO is suggested to occur through its interaction with specific cell compounds; these compounds may be found or increased in Gram positive rather than in Gram negative bacteria.[31] Another hypothesis is some components found in Gram negative bacteria, and not in Gram positives, which can oppose ZnO attachment onto cell walls; the possible nominees include the extra outer membranes and the pathogen-associated molecular patterns which include lipopolysaccharide (consisting of lipid A, core polysaccharide and O antigen), porins and particular fragments of peptidoglycan. The third supposition is that the Gram negatives cell wall, according to its structure and thickness, may prevent ZnO from penetrating into the cells and interacting with their internal components. However, further studies are required to investigate the definite interactions between ZnO particles and each individual cell component to clarify the exact antibacterial mechanism.

4. Conclusions

Synthesis of zinc oxide nano particles was achieved by using zinc sulphate and sodium hydroxide by precipitation method. Effect of calcination temperature and time on particle size was observed. It was noted that both factors were important and had effect on size, shape and antibacterial properties of the particles. Sample A and B both were synthesized through same route with different calcined temperatures and time. The detailed structural characterizations using XRD of both samples demonstrated that products were crystalline in structure and sample A had smaller particle size as compared to sample B. Moreover, SEM result showed that particles in sample A were aggregated mainly due to the large specific surface area and high surface energy. This aggregation occurred probably during the process of calcination. Both the samples were tested for antibacterial activity and it was concluded, regarding the obtained results, that ZnO nanoparticles could be proposed as an effective and powerful antibacterial agent against both Gram positive and negative bacteria. The antibacterial action was more vigorous in sample A against Gram positive bacteria than sample B. The MIC and MBC values of sample A with smaller particle size were more promising than sample B with relatively larger particle size. The application of ZnO nanoparticles could be recommended as antibiotics, preservative agent against food borne pathogens, in food production and processing, after confirming either its biosafety or toxicity.

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Povzetek

Nanodelci so deležni vse večje pozornosti javnosti, še posebej zaradi njihove potencialne uporabe v medicini. V tej študiji smo raziskovali antibakterijsko delovanje nanodelcev ZnO različnih velikosti. Nanodelce ZnO smo sintetizirali s konvencionalno metodo precipitacije z uporabo cinkovega sulfata in natrijevega hidroksida kot prekurzorja, ki ji je sledila kalcinacija precipitatov pri 350 °C 6 ur (vzorec A) in pri 550 °C 2 uri (vzorec B). Vzorce smo karakterizirali z rentgensko praškovno analizo, morfologijo delcev pa smo ocenili z vrstično elektronsko mikroskopijo. Antibakterijsko aktivnost nanodelcev ZnO v različnih koncentracijah proti štirim različnim mikroorganizmom smo ocenili z določitvijo minimalne inhibitorne koncentracije (MIK), minimalne baktericidne koncentracije (MBK) in cone inhibicije. Antibakterijska aktivnost je direktno proporcionalna koncentraciji in obratno sorazmerna velikosti delcev v primerih vseh mikroorganizmov. Antibakterijska aktivnost je večja v primerih Gram pozitivnih bakterij kot v primerih Gram negativnih bakterij. Stabilnost nanodelcev ZnO v kombinaciji z močno antibakterijsko aktivnostjo odpira možnost njihove uporabe v medicini.