Differential Susceptibility of Gram Positive and Gram Negative Bacteria Towards ZnO Nanoparticles

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Abstract

In this study, we investigate the antibacterial activity of bulk ZnO and ZnO nanoparticles, annealed at various temperatures. ZnO was prepared in the molar ratio of 1:2 using Zinc acetate dehydrate and Oxalic acid. After drying, the nanoparticles were annealed at 200^{o}C, 400^{o}C and 600^{o}C, to study the comparative size dependent activity of ZnO nanoparticles and the bulk. The products were characterised by X-Ray diffraction (XRD) analysis and size of nanoparticles were calculated to be 30 to 60 nm. UV-Vis spectroscopy measurement of absorption and band gap calculation result confirms the formation of ZnO nanoparticles. Raman spectroscopy shows that the ZnO particles have phonon at around 329 cm^{-1}, 380 cm^{-1} and 436 cm^{-1} respectively. The remaining phonon modes that occur in bulk have not been building up for particles that is a small as the ones in this study. ZnO is of particular interest due to extensive applications of the material in the personal care and home care products and recently renewed interest as a band gap engineering material. ZnO has also been shown to behave differently towards microorganisms from other metal oxides. Thus the need of hour is to develop materials using nanotechnology having bactericidal effect on broad spectrum.

In the present study bacteriological tests were performed on two Gram-positive and two Gram negative bacteria with different concentration of ZnO bulk and ZnO nanoparticles. The tests were conducted using disk diffusion and well method in Luria – Beratani and nutrient agar media on solid agar plates. Optical density of culture is maintained to 0.1 at 610 nm. Zone of inhibition was measured with 1%, 5% and 10% concentrations. Our bacteriological study showed the enhanced biocidal activity of ZnO nanoparticles compared with bulk ZnO in repeated experiments. This demonstrated that the bactericidal efficacy of ZnO nanoparticles increases with decreasing particle size and increasing powder concentration. The dominant mechanism are found to be either or both the abrasiveness i.e. physical interactions between the nanoparticles and the biological cells and the chemical interaction between hydrogen peroxide and membrane protein.

Key Word: nanoparticles, X-ray diffraction, Raman Spectroscopy, UV-Vis spectroscopy, Luria –Bertani

Introduction

The number of infections associated with antibiotic-resistant bacteria is increasing every year, thus a new approach for the development of antibiotic is necessary. Nanoparticles, which rely on entirely different mechanisms of antibacterial activity than traditional antibiotics, provide a compelling alternative

[1]. Antibacterial agents are of relevance to a number of industrial sectors including environmental, food, synthetic textile, packaging, health care, medical care as well as construction and decoration. They can be broadly classified into two types, organic and inorganic. Organic antibacterial materials are often less stable particularly at high temperature and pressure compared to inorganic antibacterial agents [2]. As a consequence, inorganic materials such as metals and metal oxides have attracted attention due to their ability to withstand harsh process conditions [3]. Among the inorganic metal oxides TiO2, ZnO, MgO and CaO are found to be stable and generally safe to human beings and animals [4]. Metal Oxides such as TiO2 and ZnO have been2 extensively used in the formulation of personal care products [5], one of the reasons may be because of their absorbance in the near ultraviolet region of spectra. ZnO has a wide direct band gap of 3.37 eV and a hexagonal wurtize structure. ZnO has recently renewed interest associated with its piezoelectricity and band gap in the near ultraviolet also a large exciton binding energy of 60 meV that could lead to lasting action based on excition recombination even at the room temperature [6].

This work is concerned about synthesis of ZnO nanoparticles without using capping agent or a surfactant. Capping agents such as thiophenol [7] and mercapto acetate [8] are toxic chemicals and are difficult to completely remove from the surface of the synthesized nanoparticles, thus making it difficult for the large scale production of nanoparticles that could contaminate the environment. Thus the comparative study of antibacterial activity of ZnO nanoparticles and bulk against Escherichia Coli , Bacillus Subtilis ,Staphylococcus aureus and Klebshiella . Therefore, the objective is to study the structural and optical properties of the product and to provide more experimental evidence on the comparative antibacterial activity that could lead to a thorough understanding of the mechanisms using a standard microbial method.

Experimental

Chemicals : Zinc Acetate dehydrate , Sodium Hydroxide and Hydrochloric acid were obtained from Merck chemical company , while absolute Ethanol was acquired from BDH Chemicals , Oxalic acid dehydrate from R and M chemicals and Ammonia solution from J.T Bakers , for comparative study Zinc Oxide was taken from Merck. All chemicals were used without further purification. Deionised water was used for the preparation of all solution as well as other respective dilutions.

Preparation of ZnO nanoparticles : Three major routes have been explored for designing the nanostructures via, reverse micellar route, surface functionalization and hydrothermal process. In surfactant aggregates “reverse micelles” contains aqueous core which acts as a micro reactor. The presence of surfactant helps in controlling the growth of the particles. Surface – functionalized method is known for the synthesis of core – shell nanorods using citric acid as a surface - functionalized agent.

Hydrothermal (Solvothermal) method is a low temperature, wet chemical route for synthesis of highly crystalline products (advantageous to increase the photo catalytic efficiency of the material). Homogeneous and less aggregated products can be obtained using this method [9].

In our study, the ZnO nanoparticles were synthesized using the sol-gel method making use of the Zinc Acetate and Oxalic Acid Dehydrate as the starting materials. Zinc acetate and Oxalic acid mixtures were prepared in ethanol with varying molar ratios of 1:1, 1:2 and 1:3. Oxalic acid was then mixed with ethanol in a beaker and stirred with a magnetic stirrer at temperature of $45^{o}\pm 5^{o}$ C for 30 min. The zinc acetate solution (ethanol + 10% volume of water) was heated at 65 ± 5^{o} C under the reflux condition for 30 min. the oxalic acid solution was then slowly added drop wise to the Zinc acetate solution under vigorous magnetic stirring to finally obtain a gel. When the gel was completely formed, the mixture was continuously stirred for an additional 60 min. The final pH of the reactant mixture was kept at 3.0-5.0± 0.2 by adding required amount of Hydrochloric acid (1.0M) and Ammonia solution respectively.

The resulting gel was dried at 80^{o} C to form the precursors for the ZnO nanoparticles (xerogel) and portions of the zinc oxide nanoparticles were taken and thermally treated at calcinations temperature of 200, 400 and 600^{o} C for 2 hrs.

Characterization of ZnO nanoparticles

The crystal sizes of the samples were determined using X-ray diffraction with Cu Kα radiation (1.5406 A^{o}) in the 2θ scan range of 20-80 ^{o}. The average crystal size of the ZnO particles was determined from XRD pattern using Debye Scherer equation:

$D=K \lambda/\beta \cos \theta$, where K is the Scherer constant (K=0.89), λ is the X-ray wave length, β is the full width at half maximum and θ is the Bragg's diffraction angle [10]. To get various sizes of ZnO nanoparticles and to analyse the antibacterial effect on size, we annealed the sample in three different temperatures. The structural characterisation of the sample and bulk was carried out by detecting the mode of vibration in Raman spectroscopy. Raman spectra were recorded by using Reins haw micro-Raman spectrometer (Re-04) equipped with solid state laser with the diode pumped at 514 nm. The band gap of the nanoparticles were determined by measuring the UV-Vis absorption spectra (in diffused reflectance mode), using Carry 500 UV-Vis NIR spectrophotometer, in the range of 200-800 nm.

Bacterial cultures and evaluation of antibacterial activities

For broad range antibacterial activity two Gram negative and two Gram positive bacterium was selected as the target organism E.coli, S.aureus, Bacillus and Klebshiella. All the dishes were sterilized in an autoclave before the experiments. Luria Beratani (LB) broth and nutrient agar were used as source for culturing the bacteria at 37^{0}C in an incubator. Optical density of the culture was maintained 0.1 at 610 nm. The Petri plates used in the tests were prepared using a nutrient agar medium. The bacteria were

sprayed evenly on the top using a sterile glass rod .The bacteria were allowed to dry (5-10 min) test solutions of ZnO of 1%, 5% and 10% concentrations and annealed at 200,400 and 600^{o}C and bulk were dropped in the well and also within the disks. The zone of inhibition was measured after 24 hrs. Incubation. Negative and positive control tubes contained only inoculated broth and free ZnO solution respectively.

Result and discussion

Preparation of nanocrystalline ZnO

Several methods of preparing nanosized ZnO powder are reported such as spray pyrolysis [11], precipitation [12], thermal decomposition [13], hydrothermal synthesis [14] and electrochemical growth [15]. Different method yield different particle size of ZnO depending on the type of precursor, the solvent, the pH, the Oxygen pressure and the temperature of the reacting solution. The choice of method depends on the final application. In this study we had chosen the sol-gel technique. Zinc acetate dehydrate and Oxalic acid in the molar ratio of 1:1, 1:2, 1:3 maintained pH between 3.0-5.0 ± .2 were prepared and annealed at 200 ,400 and 600^{0}c. The TGA-DSC spectrum shows that the final ZnO product was stable up to a temperature of 800^{0}c.No weight loss was observed at temperature beyond 420^{0}C.indicating that the product, which is a crystallized ZnO nanoparticles as confirmed via XRD analysis [16].The chemical reaction that may have taken place is ;

$$Zn(CH_3COO)_2 .2H_2O + H_2C_2O_4 .2H_2O \rightarrow ZnC_2O_4.XH_2O + CH_3COOH + 2H_2O$$

$$ZnC_2O_4.XH2O \rightarrow ZnO + CO + CO2 + X H_2O$$

Characterisation of nanocrystalline ZnO.

X-ray diffraction

The diffraction pattern of figure 1 and the inter planar spacing closely matches with those in the standard diffraction of wurtzite ZnO phase.

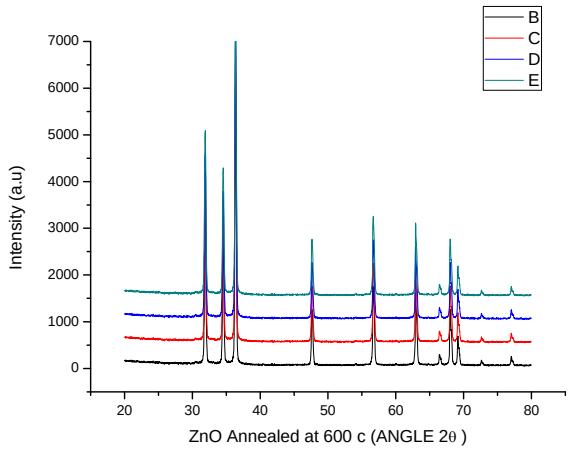

Figure 1 X-ray diffraction pattern of ZnO annealed at 200,400 and 600^{0}C and bulk.

At calcinations temperature of 6000C the lattice constants of a=b=3.2417 A0 and c=5.206 A0.The 2θ characteristics peaks of ZnO at 31.860, 34.580, 36.340, 47.660,56.760,62.9600, 66.60, 68.040, 69.120.corresponds to the (100),(002), (101), (102), (110), (103), (200), (112), (201),planes respectively of the crystal lattice. These values agree well with those provided by the standard card JCPDS 36-1451. Debye Scherer equation is used to calculate the crystalline size in the range of 30-60 nm.With increasing annealing temperature the crystallinity of the sample appears to improve and the grain size is also increased, reflecting the effect of temperature on the crystalline size. The effect of pH on the synthesis of ZnO nanoparticles was taken care and for smallest crystal particle size at optimal molar ratio of 1:2 and pH of 2 ± 0.2 was found and at the same time particle of size 40 nm were found when pH was above 3[17,18].

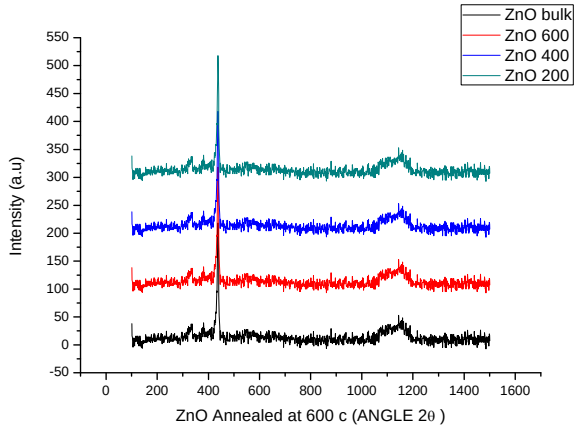

Figure 2 Raman Vibrations

Raman Spectroscopy

The spectrum obtained from Raman Scattering measurement is used as a fingerprint for the identification of species and crystal phases along with that, it also gives information of the vibration spectra of the entire molecule or the crystal understudy.

In Figure 2 the comparative Micro Raman Spectroscopy was recorded at 514 nm diode pumped. Optical Phonon vibrations were recorded at nearly 329 nm, 380 nm and the most important peak at 436 nm which identifies the formation of ZnO nanoparticles, remaining phonon vibrations were not observed in these samples which are reported for bulk [19]. This confirms that when size of a crystalline particle decreases, sum of the phonons or vibrational modes can be restricted due to confinement in the small volume of the particle and manifest itself in shifting the vibrations to different energies, changes in the symmetry of the vibration peaks and peaks can be extinct or even enhanced. Thus the peak shift may be due to at least two different mechanisms, spatial confinement within the dot boundary and phonon localisation by defects [20].

3.2.3 UV-Vis spectroscopy

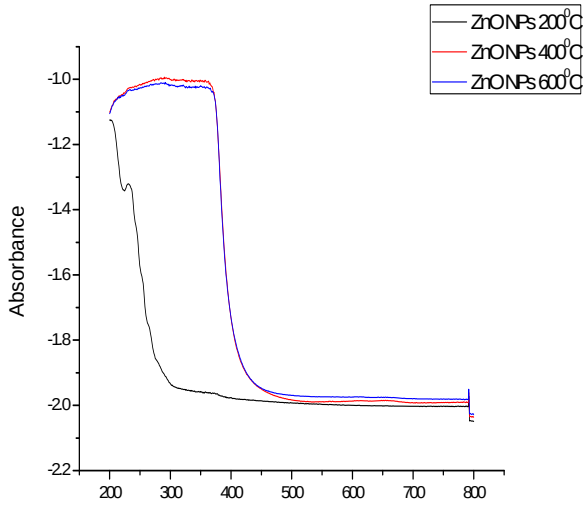

Fig. 3. UV-Vis spectroscopy

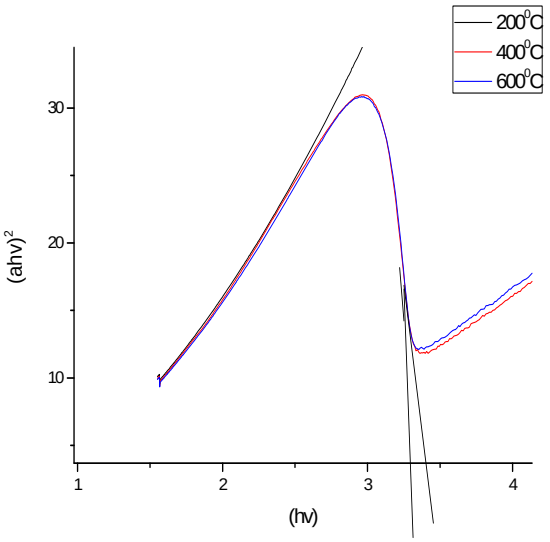

Fig.4. Tauc plot

The optical absorption properties of the various samples were measured by UV-Vis absorption spectra (in diffuse reflectance mode) in the range of 200-800 nm. It is clearly visible from the spectra that the tail of absorption edge is towards the visible region.

The sample annealed at 200o C is showing some different spectra confirming that the TGA-DSC analysis which shows that ZnO nanoparticles are not stable below 420o C [21]. With increasing annealing temperature the spectra is more stable. It is known that ZnO is a direct band gap material and the energy gap (Eg) can be estimated by assuming direct transition between conduction band and valance band. Theory of optical absorbtion gives the relationship between the absorbtion coefficient α and the photon energy hγ for direct allowed transition as

$(\alpha h\gamma)^2 = A(h\gamma - Eg)$, where A is a function of the index of refraction and whole/electron effective mass. The direct band is determined using this equation when the linear portion of the $(\alpha h\gamma)^2$ is plotted against hγ intersect the energy axis at α = 0[22]. The band gap is 3.42 ev.

Evaluation of antibacterial properties.

The ability of antibacterial agent to rupture bacterial cell is tested by disk diffusion as well as well diffusion method. The effect was more significant in well diffusion and thus reported. Here ZnO nanoparticles annealed at three different temperatures with three different concentrations were tested on two Gram positive and Gram negative bacteria. The presence of an inhibition zone clearly indicates the

biocidal action of Zno nanoparticles. We noticed that the smaller particles are more effective. This can be explained on the basis of the oxygen species released on the surface of ZnO nanoparticles, which leads to the fatal damage to the microorganism [23, 24]. The generation of highly reactive species such as OH^-, H_2O_2 and O_2^{2-} is explained as follows

Table no. 1 comparative antibacterial activity

Sr.	Bacteria	Annealing temperature	ZOI at concentration		
			1%	5%	10%
1.	Escherichia coli	200^0C	18 mm	20 mm	21 mm
		400^0C	14 mm	17 mm	19 mm
		600^0C	12 mm	14 mm	16 mm
		Bulk	12 mm	18 mm	22 mm
2.	Bacillus subtilis	200^0C	14 mm	16 mm	17 mm
		400^0C	11 mm	14 mm	15 mm
		600^0C	10 mm	12 mm	14 mm
		Bulk	10 mm	11 mm	12 mm
3.	Staphylococcus aureus	200^0C	19 mm	21 mm	23 mm
		400^0C	19 mm	20 mm	23 mm
		600^0C	18 mm	19 mm	22 mm
		Bulk	8 mm	11 mm	15 mm
4.	Klebshiella	200^0C	10 mm	11 mm	13 mm
		400^0C	9 mm	10 mm	12 mm
		600^0C	---	2 mm	3 mm
		Bulk	---	----	---

Since ZnO with defects can be active by both UV and visible light, electron-hole pairs ($e^- h^+$) can be created. The hole split H_2O molecules (from the suspension of ZnO) into OH^- and H^+. Dissolved oxygen molecules are transformed to super oxide anions ($*O_2^-$), which in turn react with H^+ to generate (HO_2*) radicals, which upon subsequent collision with electrons produce hydrogen peroxide anions (HO_2^-). They then react with hydrogen ions to produce molecules of H_2O_2. The generated H_2O_2 can penetrate the cell membrane and kill the bacteria [25, 26].

$$ZnO + hv \rightarrow e^- + h^+ \quad ; \quad h^+ + H_2O \rightarrow *OH + H^+ \quad ; \quad e^- + O_2 \rightarrow ®O_2^-$$

$$®O_2 + H^+ \rightarrow HO_2® \quad ; \quad HO_2® + H^+ + e^- \rightarrow H_2O_2$$

Since the hydroxyl radicals and super oxides are negatively charged particles, they cannot penetrate into the cell membrane and must remain in direct contact with the outer surface of the bacteria, however H_2O_2 can penetrate into the cell [27]. Thus the cell membrane ruptures and the lipid flows out killing the cell. The generation of H_2O_2 depends on the surface area of ZnO, which results in more oxygen species on the surface and the higher antibacterial activity of the smaller nanoparticles. Therefore the bulk ZnO showed less bacterial activity than the nanoparticles. When the particles are small more number can accumulate on the bacteria, killing it faster than the bigger particle. This is the chemical explanation. The other cause may be the physical structure due to which the abrasive surface may rupture the cells leading, it to the fatal damage [28, 29, 30].

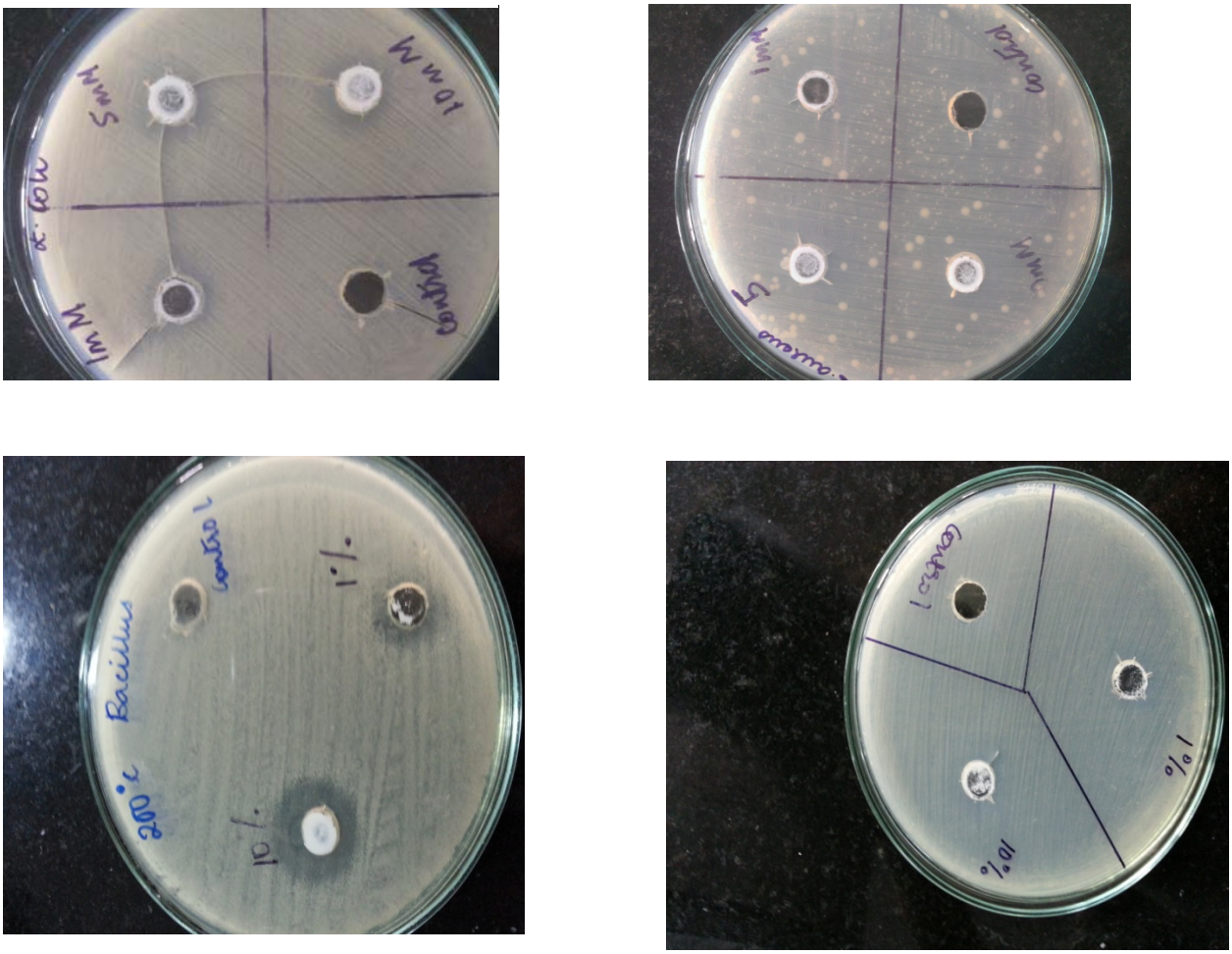

Fig 5. Plates with different bacteria showing zone of inhibition

Conclusions

ZnO nanoparticles using sol-gel method were synthesized with various molar concentrations maintained different ph values and annealed at different temperature. The minimal size of nanoparticles could be obtained with molar ratio of 1:2 at pH 2.0 ± 0.2 and calcinations temperature 400^0C. Bioactivity of ZnO nanoparticles compared to bulk was studied using various concentrations. The enhanced bioactivity of smaller particles is attributed to the higher surface area to volume ratio, resulting in more generation of active oxygen species, killing bacteria more effectively than the bulk. The dominant mechanism is found to be either or both the abrasiveness that is the physical interactions between the nanoparticles and biological cells and the chemical interaction between the hydrogen peroxide and cell membrane. This study can be used as toxicity of metal oxide nanoparticles as biocides or disinfectants.

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