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Comparison of antimicrobial and anticancer activity of ZnO nanoparticles prepared using different precursors by hydrothermal synthesis

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ABSTRACT

In our present work, we evaluate the effect of precursors on the synthesis of ZnO nanoparticles (NPs) by novel hydrothermal method. The structure and morphology of the samples were subjected to extensive investigations by PXRD, FTIR, FESEM and HRTEM with respect to the determination of the formed phases and morphology. Surface area measurement of the samples was carried out by standard Brunauer-Emmett-Teller technique. Antibacterial response of the samples prepared using two different alkali was carried out against *Clostridium perfringens* and *Salmonella enterica* by well diffusion method. In vitro anticancer efficacy of the NPs has been tested on HeLa cell lines by 3-(4,5-dimehtylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

KEY WORDS: ZnO nanoparticles, Hydrothermal, Antibacterial, MTT assay.

1. INTRODUCTION

The continuous development of antibiotic resistant pathogen species has brought about the need for new antimicrobials. Inorganic metal oxides like TiO_2 and ZnO are already being widely used in sunscreens, cosmetic products (Mehdi Ansari, 2013; Newman, 2009), textiles, antimicrobial coatings (Petya Petkova, 2014) etc. The antimicrobial properties of nanoparticles (NPs) depend significantly on their size, surface area, composition, surface charge and shape (Angelique Simon-Deckers, 2009). The antibacterial response of ZnO has been studied with different bacteria such as *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus pyogene* and fungi such as *Botryis cinera*, *Penicillium expansum*, *Candida albicans* etc.

ZnO is a wide band gap semiconductor with an energy gap of 3.37 eV at room temperature, that has antimicrobial activity and it is generally recognized as safe (GRAS) under US-FDA listings, to human beings and animals. ZnO is regarded as a material which is environmental friendly that has important biomedical applications related to bio imaging and cancer detection. ZnO is a magic material as it possesses wide range of applications and flexibility of preparation in different morphologies with different properties. Introduction of porosity in ZnO based nanostructured materials can greatly enhance their surface property. ZnO has gained much importance as it can be applied in many applications such as for gas sensing, photo catalyst, UV photo detection, piezoelectric nano generators, solar cells as well as medicinal applications. They are well known for UV sensors, as a polymer joining material. ZnO structures posses' transparent-conductive activity too. The action of ZnO structures against Hepes simplex virus type 1 and type 2 has also been studied (Antoine, 2012). ZnO NPs have gained much attention in the area of cancer therapy. The cytotoxicity of various shped ZnO NPs with varying particle sizes have been tested on cancer cells such as HepG-2, HeLa (Wahab, 2009; 2014), U87 (Wahab, 2011), HCT 116, PC-3, A549 (Prashanth Gopala Krishna, 2016; Prashanth, 2015), MCF-7 (Prashanth, 2015) etc. However, at nanoscale the material still needs extensive investigation before industrialization.

Hydrothermal method is proved to be the most convenient procedure due to its ability to control the particle size by controlling the synthesis conditions such as temperature, time, etc. (Xiu, 2013). The hydrothermal synthesis of ZnO NPs has many advantages like powders with nanometer-size can be obtained, the reaction can be carried out under moderate conditions, powders with different morphologies can be generated by regulating the reaction conditions, and the as-synthesized powders have been recognized to possess unique properties from that of the bulk (Lin Tan, 2011; Moulahi, 2013). The effect of concentration of the precursors, temperature and time of growth on the structure, grain size of ZnO NPs by hydrothermal method has also been reported (Aneesh, 2007). In our previous studies we have shown the selective anticancer activity of ZnO nanopellets synthesized by hydrothermal method (Prashanth Gopala Krishna, 2016).

The present study is focused on the synthesis of ZnO NPs by hydrothermal method using two different alkali namely NaOH and KOH and examination of the effect of nature of the alkali and time of growth on their properties. Further to the synthesis of ZnO NPs, our interest was to investigate their antibacterial and anticancer response. The

interaction of NPs with microorganisms and biomolecules is an expanding area of research, which is still largely unexplored yet. Thus, with this background our present study reports the synthesis of ZnO NPs using different alkali by simple, convenient, low temperature hydrothermal method and evaluation of their antimicrobial activity against Gram-positive bacterium *Clostridium perfringens* and Gram-negative bacterium *Salmonella enterica* by well diffusion method. In vitro anticancer studies have been carried out against HeLa cells by (MTT) assay.

2. MATERIALS AND METHODS

Materials/ Chemicals: Zinc nitrate hexahydrate [$Zn(NO_3)_2.6H_2O$, AR 99% SD Fine], Sodium hydroxide [NaOH, AR 99% SD Fine], Potassium hydroxide [KOH, AR 99% SD Fine], Nutrient agar [Himedia], Dulbecco's Modified Eagle's medium [Gibco], MTT [$C_{18}H_{16}BrN_5S$, 97.5%, Sigma Aldrich], Dimethyl sulfoxide [C_2H_6SO, AR 99% Merck], were used as such without further purification.

Synthesis of ZnO NPs: The synthesis of ZnO NPs was carried out by hydrothermal method as described in our previous work (Prashanth Gopala Krishna, 2016). 7 g of Zn $(NO_3)_2.6H_2O$ was dissolved in 40 mL of double distilled water. A solution of 1.0 N NaOH was added till, pH of the solution reached 12. The solution was agitated for 15 minutes, transferred in to a 100 mL Teflon-lined stainless steel autoclave, sealed and was maintained at an external temperature of 180 ˚C for 12 h. After that, the autoclave was allowed to cool naturally to room temperature. The ensuing precipitate was washed with double distilled water many times and then with absolute ethanol, to get rid of the ions adhering to the final product. The product was dried at 110 ˚C in hot air oven for 2 h and further cooled to room temperature. The sample was labeled as ZnO-1. The same procedure was repeated for 16 h and the sample was labeled as ZnO-2.

Similar method was followed using KOH as alkali. ZnO NPs thus synthesized were labeled as ZnO-3 and ZnO-4 for the reactions carried out for 12 h and 16 h respectively.

Characterizations: ZnO NPs synthesized were characterized by various standard characterization tools. The crystal structure of the samples was recorded using Panalytical X'pert diffractometer with Cu Kα radiation (λ=1.5418 Å) as the source. The formation of ZnO and the absence of any other functional groups from the precursors were confirmed using fourier transform infrared spectroscopy (FTIR) by KBr disc method using Perkin-Elmer spectrophotometer (Model: Spectrum 1000) within the range of 350-3000 cm^{-1}. The morphology of the sample was characterized by Field Emission Scanning Electron Microscopy (FE-SEM) performed on FEI Quanta FEG 200 - High Resolution Scanning Electron Microscope. The shapes were explored by High Resolution Transmission Electron Microscopy (HRTEM) carried out on JEOL 3010. Brunauer-Emmett-Teller (BET) surface area measurement was carried out on Micromeritics ASAP 2020.

Evaluation of antibacterial activity: Homogeneous dispersions of NPs with 2 fold concentrations varying from 1 mg/mL to 250 µg/mL were prepared by ultrasonication. 20 mL of sterilized, molten and cooled nutrient agar media was poured in the sterilized petri dishes. The bacteria *Clostridium perfringens* and *Salmonella enterica* were cultured overnight at 37°C in nutrient agar and adjusted to a final density of 10^7 CFU/mL by 0.5 McFarland standards. 100 µL of the pathogenic bacteria cultures were transferred onto plate and made culture lawn by using sterile L-rod spreader. Wells were cut and dispersions of ZnO NPs of distinct concentrations were inoculated into them. The plates were then incubated at 37°C for 24 h. The antibacterial activity of NPs was determined by measuring the diameter of the zone of inhibition formed around the wells. Ofloxacin was used as the positive control.

Anticancer activity by MTT assay: Anticancer activity of ZnO NPs was carried out by MTT assay as explained in our earlier studies with minor modifications (Prashanth Gopala Krishna, 2016). HeLa cell lines (procured from ATCC) of 80% confluent were trypsinized. The viable 50,000 cells/well were seeded in a 96 well plate and incubated for 24 hr at 37°C, 5 % CO_2 incubator. ZnO NPs from 0-320 µg/mL in Dulbecco's Modified Eagle's medium without fetal brovine serum were incubated for 24 h. After incubation with ZnO NPs the media was removed from the wells and 100 µL/well of the MTT (5 mg/10mL of MTT in 1 × Phosphate buffered saline, the solution was filtered through 0.2 µM filter) working solution was added and incubated for 3 to 4 h. After incubation with MTT reagent, the media was removed from the wells and 100 µL of DMSO was added to rapidly solubilize formazan and absorbance was measured at 590 nm. Percent of inhibition was calculated as [100-(As/Ac) × 100] and cell viability was calculated as [As × 100/Ac] where, as and Ac are the absorbance values of the sample and control respectively.

3. RESULTS AND DISCUSSION

X-ray diffraction patterns: The PXRD pattern of ZnO-1, ZnO-2, ZnO-3 and ZnO-4 synthesized using NaOH and KOH as alkali is presented in Figure 1 (a-d) respectively. The results were examined with Crystallographica Search-Match (CSM). PXRD of all the samples showed the crystalline nature of the sample having hexagonal structure with the standard Joint Committee on Powder Diffraction Standards (JCPDS) No. [36-1451] corresponding to zincite pattern. Hence they can be indexed as hexagonal wurtzite type of ZnO.

FTIR analysis: FTIR spectra of ZnO-1, ZnO-2, ZnO-3 and ZnO-4 are depicted in Figure 2 (a-d) respectively. The absorption bands at 1400-1515 cm^{-1} were likely related to absorption of atmospheric CO_2 on the metallic cations.

The transmittance bands at 470 cm^{-1} and 416 cm^{-1} correspond to the Zn-O bonding and confirm the presence of ZnO particles. The FTIR results confirm the high purity of ZnO NPs.

Morphological studies: The surface morphology of ZnO NPs was studied using the scanning electron microscopy. The FE-SEM micrographs of the samples are shown in Figure 3 (a-d). These micrographs reveal that the particles of ZnO-1 and ZnO-2 have non-uniform pellet like morphology, particles of ZnO-3 and ZnO-4 have flake shaped morphology. Figure 3 (e-h) shows the HRTEM images of ZnO NPs. It confirms that the ZnO-1 and ZnO-2 particles are pellet and rod shaped and ZnO-3 and ZnO-4 particles are flake and rod shaped with non-uniform thickness and diverse shapes.

Surface area measurements: The surface area of ZnO NPs was measured by the standard BET technique with N_2 adsorption-desorption isotherms on Micromeritics ASAP 2020. Degassing condition was 120°C outside the instrument, 1 h in-situ degassing at 120°C and degassing outside the instrument was for 24 h. The BET surface area values of the samples are presented in Table 1. These results indicate the higher surface area of the samples prepared using NaOH as alkali over the samples prepared using KOH as alkali maintaining the same reaction conditions.

Antibacterial activity: The antimicrobial results of ZnO NPs on different organisms are presented in Table 2. As observed from the results, the zone of inhibition is maximum at 1 mg/mL indicating that at higher concentrations the ZnO NPs are exhibiting antimicrobial properties. The results show that the zone of inhibition is maximum for ZnO-2 against both the organisms than ZnO-4. The specific mechanism of the bioactivity of ZnO is still under discussion. Several mechanisms have been proposed to explain the antibacterial activity of ZnO NPs : (a) One of the possible mechanisms is based on the abrasive surface texture of ZnO- binding of ZnO NPs to the bacterial surface is due to electrostatic forces that directly kill bacteria (Stoimenov, 2002), (b) mechanical destruction of the cell membrane caused by penetration of the nanoparticles (Brayner, 2006) (c) release of Zn^{2+} ions from the nanoparticles (Heinlaan, 2008) and (d) active oxygen generated from the powder (Xu, 2003; Zhang, 2007; Yang, 2009; Akhavan, 2009; Franklin, 2007). Hence, two important possible mechanisms involved in the interaction between NPs and bacteria suggested by several investigations are (a) the production of increased levels of ROS, mostly hydroxyl radicals and singlet oxygen (Heinlaan, 2008; Yang, 2009; Akhavan, 2009; Franklin, 2007; Anat Lipovsky, 2009) and (b) Zinc toxicity of cell membrane by adhesion of ZnO particles which inhibits the bacterial growth (Xu, 2003; Zhang, 2007). Antimicrobial activity depends on the surface area. This factor has been often proved by many studies (Amornpitoksuk, 2011; Lingling Zhang, 2008). In our present studies ZnO-2 exhibited higher antimicrobial activity than ZnO-4 against the test organisms. This finding might be due to higher surface to volume ratio of ZnO-2 than ZnO-4. The results also demonstrate that the zone of inhibition is maximum for ZnO NPs against Gram-positive bacterium compared to Gram-negative bacteria tested in our studies. These results are in well agreement with the literature which reports that the ZnO NPs effect is more pronounced against Gram-positive bacterial strains than Gram-negative bacterial strains (Premanathan, 2011; Ameer Azam, 2012). These results also suggest that ZnO NPs are not only toxic to Gram-positive bacteria, but also to Gram-negative bacteria.

Anticancer activity: The results of cytotoxic effect of ZnO-2 and ZnO-4 against HeLa cells are shown in Figure.4(a). Dose dependent response showing decrease in cell viability with increase in ZnO NPs concentration was observed. IC_{50} value, the concentration of ZnO NPs needed to inhibit cell growth by 50% for cytotoxicity test on HeLa cells were derived from nonlinear regression analysis (curve fit) based on sigmoid dose response curve (variable) and computed using Graph Pad Prism 5 (Graphpad, San Diego, CA, USA). It is shown in Figure.4(b). The IC_{50} values are found to be 41.85 µg/mL and 137.6 µg/mL for ZnO-2 and ZnO-4 respectively. These studies indicate the higher anticancer activity of ZnO-2 than ZnO-4. This might be due to the higher surface area of ZnO-2 than ZnO-4. Studies have shown that ZnO NPs induce cytotoxicity in a cell specific and proliferation dependent manner by rapidly dividing cancer cells being the most susceptible and quiescent cells being the least sensitive (Premanathan, 2008; Cory Hanley, 2008). However, the anticancer activity of ZnO NPs, in particular the mechanism of apoptosis in cancer cells due to ZnO NPs is still not clear.

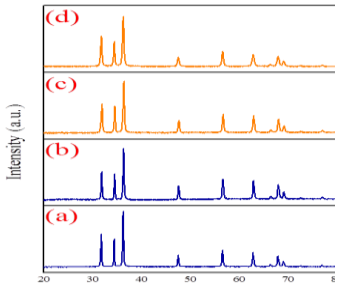

Figure.1. (a-d): PXRD pattern of ZnO-1, ZnO-2, ZnO-3 and ZnO-4 respectively

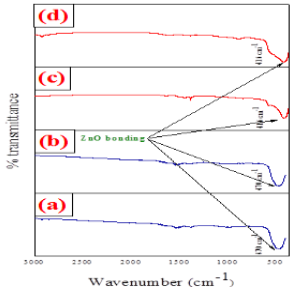

Figure.2. (a-d): FTIR spectra of ZnO-1, ZnO-2, ZnO-3 and ZnO-4 respectively

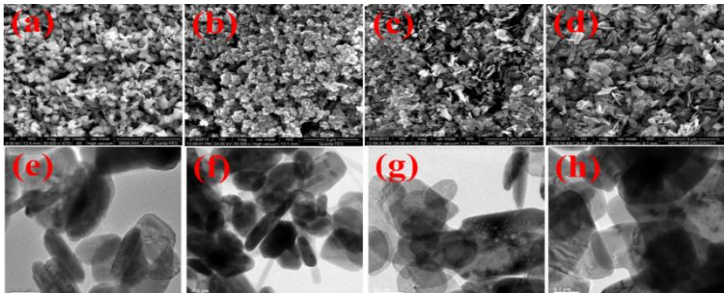

Figure.3. FE-SEM images of (a) ZnO-1, (b) ZnO-2, (c) ZnO-3, (d) ZnO-4, HRTEM images of (e) ZnO-1, (f) ZnO-2, (g) ZnO-3 and (h) ZnO-4 respectively

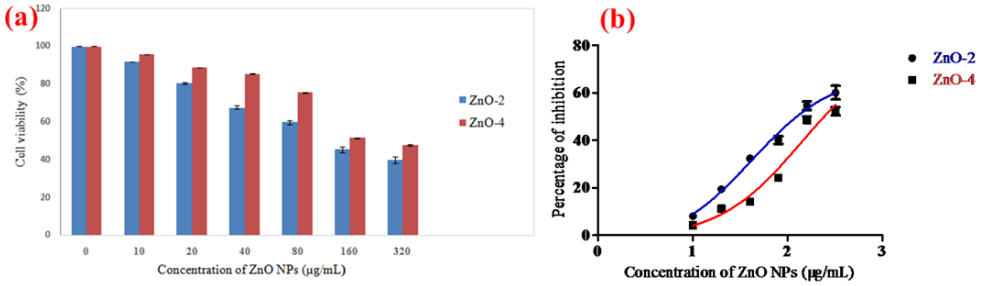

Figure.4. (a) Cytotoxic effect of ZnO NPs in HeLa cell lines. Cells were treated with various concentrations (0, 5, 10, 20, 40, 80, 160, 320 µg/mL) of ZnO NPs for 24 h grown in a serum free media. The percentage of cell death induced was determined using the MTT assay (data represent mean ± SD), (b) Determination of IC_{50} values of ZnO-2 and ZnO-4 on HeLa cell lines (data represent mean ± SD)

Table.1. BET surface area of ZnO NPs

Sample	Surface area (m^2/g)
ZnO-1	9.13
ZnO-2	22.79
ZnO-3	7.60
ZnO-4	12.85

Table.2. Results of antimicrobial activity of ZnO NPs (Zone of inhibition in mm)

Concentration of ZnO dispersions				
Sample	**1 mg/mL**	**500 µg/mL**	**250 µg/mL**	**Positive control 100 µg/mL**
ZnO-2	11.25±0.433	9.50±0.500	7.75±0.433	*Clostridium perfringens*
ZnO-4	8.50±0.500	7.50±0.500	6.00±0.829	34.25±0.500
ZnO-2	13.00±0.500	10.75±0.829	9.00±0.707	*Salmonella enterica*
ZnO-4	11.25±0.433	9.50±0.500	7.75±0.500	34.50±0.500

Values are the mean ± SE of inhibition zone in mm

4. CONCLUSION

All the samples of ZnO NPs prepared using different precursors and varying the reaction conditions by low temperature hydrothermal method exhibited hexagonal wurtzite structure. It was observed that the samples prepared using NaOH as alkali exhibited better properties in terms of surface area and in-turn showed greater antimicrobial activity against *Clostridium perfringens* and *Salmonella enterica*. MTT results indicate that ZnO NPs induce concentration dependent cytotoxicity against HeLa cells. This study concludes with a simple note that NaOH could indeed be more advantageous than KOH in the hydrothermal synthesis of ZnO NPs when scrutinizing for their antimicrobial and anticancer activity. Further experimentation on the different duration and temperature of nucleation, concentration of the alkali shall be conducted on to understand the efficacy of the alkali on the hydrothermal synthesis of ZnO NPs.

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