

Cytotoxicity of zinc oxide nanoparticles coupled with folic acid and polyethylene glycol

A. D. Khalid^a, N.-ur-Rehman^{b,*}, F. Hadi^c, S. S. Iqbal^a, S. A. Buzdar^b, A.K. Khan^b

^a*Department of Physics, The University of Lahore, Lahore, Pakistan*

^b*Institute of Physics, The Islamia University of Bahawalpur, Bahawalpur, 63100, Pakistan*

^c*Institute of molecular Biology and Biotechnology, The University of Lahore, Lahore, Pakistan*

The anticancer mediators claim their activity in liquid form as a biocompatible and use in bios stems, which are mostly collected in fluid form. Control on the stability of metal oxide nanoparticles in parallel reducing the effect of large amount on their biological and cytotoxicity activities, remains a challenge. Here anticancer activity and cytotoxicity zinc oxide nanoparticles (NPs) have been presented that are coated with folic acid (F.A) and polyethylene glycol (PEG). The nanoparticles were synthesized by wet chemical method and characterized by x-ray diffraction; scanning electron microscopy and Fourier transform infrared spectroscopy. The cytotoxicity studies were performed on HepG2 cells. MMT assay was used to check the cell viability. The PEG and F.A capped ZnO NPs have shown reduction in cytotoxicity as compare to bare ZnO nanoparticles.

(Received July 10, 2021; Accepted January 19, 2022)

Keywords: Zinc oxide, MTT assay, Folic acid, Poly ethylene glycol

1. Introduction

Since the last decade, the nanotechnology gained more interest of scientists and researchers around the world. Among these nanomaterials the metal oxides have been explored the most due to their unique chemical and physical properties, low fabrication cost, controlled particles size and high stability [1-3]. The various applications of metallic oxides in the biological field are blessings owing to their low toxicity and biocompatibility [4-6]. The zinc oxide (ZnO) nanoparticles (NPs) are more biocompatible compared to the other metal oxides as these are comparatively less toxic. For example, silver or nickel based oxide NPs raised their cytotoxicity at higher concentration in the presence of heavy elements. Secondly in the view of applications, their stability towards agglomeration is of important role [7, 8]. Stability of the NPs in the solution is their primary issue [9]. The capping agents on the NPs increases their life span in the suspension by avoiding agglomeration [10]. The cytotoxicity of various metal oxide NPs is also a concerned problem due to their effects on the environment and human health [11, 12]. The aggregation of NPs can be prevented with stabilizers and biocompatible polymers coating, which also helps decreasing their toxicity [13]. The level of toxicity of NPs can be determined through mechanism of reactive oxygen species (ROS) generation for cellular oxidative stress [14]. Elje et al. checked the cytotoxicity of bare ZnO NPs on HepG2 cells and their findings show the dependence of cytotoxicity on the concentration of ZnO NPs [15]. Jevapatarakul D., et al. synthesized ZnO NPs by green method and exposed on skin cancer cells (A431) to analyze their cytotoxicity. They also concluded that the cytotoxicity of ZnO NPs is dose and concentration dependent [16]. Khorsandi L., et al. treated MCF-7 with ZnO NPs and they reported the same observations as by the earlier references [17]. Pengyu Chen. Et al also studied the cytotoxicity on bare ZnO NPs on HepG2 cells. They concluded that the cytotoxicity depends on size of NPs and concentration [18]. Xiaoming et.

* Corresponding author: naeem.rehman@iub.edu.pk
<https://doi.org/10.15251/DJNB.2022.171.73>

al., studied the cytotoxicity of metal oxide NPs coated with EDTM P. They concluded that metal oxide could induce reactive oxygen species (ROS) generation and cell death due to oxidative stress, but coating layer reduces the surface activity of metal oxide. The EDTMP coated metal oxide showed negligible ROS generation and oxidative stress towards THP-1 and BEAS-2B cells [19]. Negvenkar et.al also reported the cytotoxicity of ZnO and CuO NPs coated with polyethylene glycol (PEG) and polyvinyl alcohol. They observed that the cytotoxicity were decreased with polymer capping of NPs toward bacteria [20]. The same results were reported by Luisa F. et al that ZnO NPs coated with PEG and PEI reduces their cytotoxicity on lungs cells [21].

The presented work focuses on the issue of ZnO NPs by capping with polymers, folic acid (F.A) and polyethylene glycol (PEG) and evaluation of their cytotoxicity towards HepG2 cells.

2. Experimental method

The nanoparticles ZnO were synthesized by the co-precipitation method by using Zinc chloride ($ZnCl_2$) and Sodium hydroxide (NaOH) + distilled water and ethanol as precursors. For the purpose, 2.725g of $ZnCl_2$ was added into distilled water to make solution of 20ml. then a sodium hydroxide solution by adding 4.8g of (NaOH) into 30 ml of distilled water. It was added drop wise into the $ZnCl_2$ solution with continuous stirring at 45°C. After mixing the solution was stirred for 2 hours which resulted in the formation of white precipitates. These ZnO precipitates were then separated out from the solution by centrifuge machine and washed with a mixture of ethanol and distilled water several times. These particles were then place in a drying oven at 100°C for 24 hours followed by the grinding process. The ZnO NPs are reserved for further processes. The prepared ZnO NPs were loaded with folic acid (FA) and polyethylene glycol (PEG) by ratio of 1:1 to obtain F.A-PEG-ZnO nano-conjugates by 1:1 volume ratio. Their procedure is described as the solution of PEG was prepared by dissolving 100 mg into 20 ml distilled water. The Prepared sample ZnO NPs were dipped into solution, sonicated at room temperature for 2 hours, washed with ethanol and dried at 80° C in the oven to get PEG functionalized samples. After that 25 mg folic acid was dispersed into 25 ml of Dimethyl sulfoxide (DMSO) and stirred. The prepared samples were added into solution; the mixture was sonicated for 3 hours, washed with distilled water and dried in oven at 60° C to obtain F.A-PEG-ZnO nanoconjugates.

The shapes and morphology of ZnO NPs were observed by scanning electron microscopy. The particle size also measured directly from SEM images. The crystalline size and phase of ZnO NPs were investigated from XRD patterns. The interaction of ZnO NPs with PEG and F.A were investigated by FT-IR spectra by using a Frontier™ FT-IR spectrometer with absorbance mode (%) was measured in the range of 600 to 3500 cm^{-1} .

2.1. Cell culturing & Cell viability assay

The human liver cancer cells (HepG2) were exposed by ZnO NPs. The cell viability was determined after exposing of samples. The cells were cultured in 95% relative humidity in atmosphere with 5% CO_2 at temperature of 37 °C. The phase contrast simple microscopy was used to check the intracellular distribution of samples and the cellular accumulation efficiency. The cancer cells were seeded onto 6-well plates with density of 6.0×10^5 cells per well and incubated at temperature of 37 °C for 24 hours. The suspension of samples was added into cancer cells and they were incubated for 24 hours again, cells were then washed with PBS (pH 7.4) at least three times after treatment with samples. The cells were suspended with PBS containing FBS (2%, v/v) after collecting the cell pellets by configuration. Finally Cells were counted according to the fluorescence intensity in each sample by a FACSCalibur fluorescence-activated cell sorter (FACS™) equipped with CellQuest software. The intracellular location of samples in cells was observed by phase contrast microscopy. MTT assay was used for Viability of HepG2 cells [22, 23] the samples were exposed on briefly, 6.0×10^5 cells/well were seeded in 6-well plates for 24 hours. The culture medium was separated from each well to avoid interference of samples (nanoparticles) after exposure, new medium consisting MTT solution (0.5 mg/mL) in an amount equal to 10% of culture volume was replaced and incubated for 3 hours at temperature of 37°C until a purple-colored formazan product formed. The final formazan product was dissolved in

acidified isopropanol. To settle down the remaining samples the 6-well plate was centrifuged at $2300 \times g$ for five minutes then, a $100 \mu\text{L}$ supernatant was transferred to other fresh wells of a 6-well plate, and at wave length of 570 nm the absorbance was measured by a microplate reader (FLUOstar Omega, Cary, NC). All The experiments were done thrice and their data was evaluated as the mean \pm standard deviation (SD). All results were conducted by using the Prism software package

3. Results and discussions

The crystalline structure of Zinc oxide nanoparticles (ZnO NPs) were investigated by X-rays diffraction (XRD) technique with Cu $K\alpha$ radiation ($\lambda = 0.15418 \text{ nm}$). Figure 1 reflects the XRD patterns of Zinc oxide NPs. The peaks at $2\theta = 31.67^\circ$, 34.31° , 36.14° , 47.40° , 56.52° were assigned to (100), (002), (101), (102), (110), of ZnO NPs. These peaks represents that the samples were a crystal of wurtzite structure (PDF JCPDS 36-1451). No peak related to impurity phase was detected, which suggested that pure zinc oxide nanoparticles with high crystalline quality have been fabricated successfully.

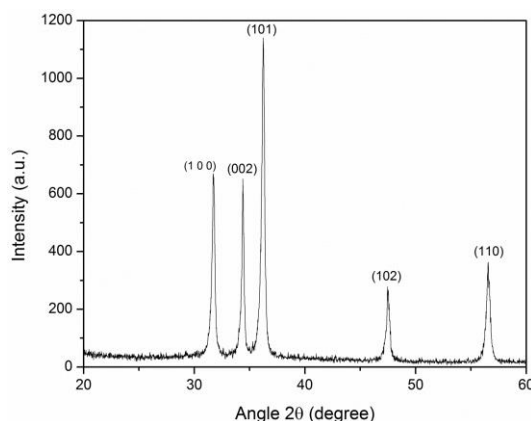


Fig. 1. XRD pattern of ZnO nanoparticles

From the XRD pattern, the crystallite size (d) of samples was measured by Scherer's formula [24]

$$d = k\lambda / \beta \cos \theta$$

here $k = 0.9$ describes the shape factor, λ is the wavelength of X-ray Cu $K\alpha$ radiations (1.54 \AA), θ is the angle of Bragg diffraction and β shows the full width at half maximum of the particular diffraction peak. The measured average crystallite size of ZnO NPs was 37.93 nm .

Figures 2 show the scanning electron microscopy (SEM) images of the ZnO NPs. The figure 2 depicts the morphology of ZnO nanostructures. These nanostructures are seen to have columnar growth pattern with a non-uniform rod length. The average diameter of these nano-rods is found to be around 40 nm .

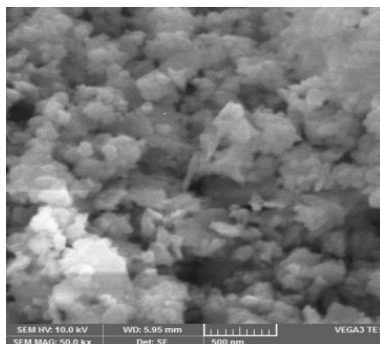


Fig. 2. SEM image of ZnO nanoparticles.

The FTIR spectra for ZnO NPs and their nanoconjugates are shown in figure 3. The figure 3(a) represents the ZnO, in that graph the peaks 687 and 743 cm^{-1} represents ZnO according to literature review the peaks between 450-800 cm^{-1} are stretching vibrations of Zn-O [25]. The figure 3(b) represents their nanoconjugates (F.A-PEG-ZnO) The peak at 670 cm^{-1} represents ZnO [25]. The peaks at 1519 and 1619 cm^{-1} shows the folic acid [26] and the peaks at 1342 and 2920 confirms the presence of PEG in sample [27].

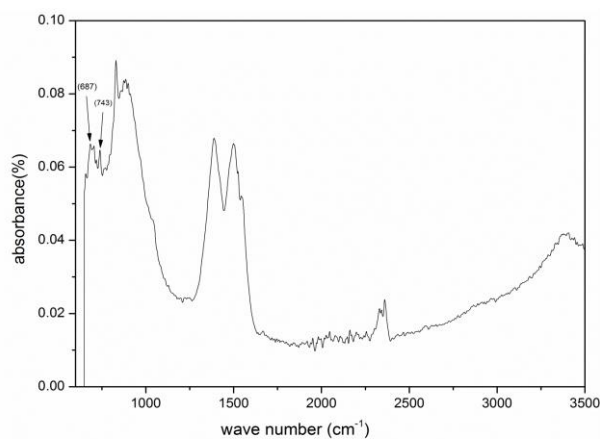


Fig. 3. (a) FTIR pattern of ZnO

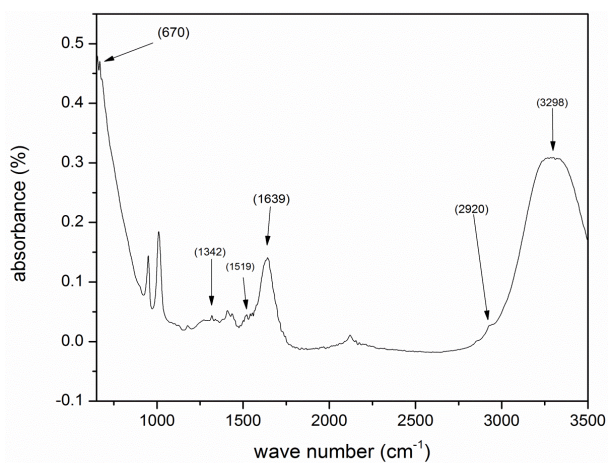


Fig. 3. (b) FTIR pattern of F.A-PEG-ZnO

3.1. Cytotoxicity of Nanoparticles

The HepG2 cells were treated with ZnO NPs, and functionalized with polymers PEG and F.A nanoconjugates (F.A-PEG-ZnO) at the different concentrations of 5 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$, and 15 $\mu\text{g/mL}$ for duration of one day to analyze their cytotoxicity, MTT assay was used to measure their cytotoxicity as shown in (Figure 4). The outcome has showed that both samples killed the cells by ROS generation which is indicated through cell viability. It has been recommended that ROS generation are the general process through which set off apoptosis in cancer cells [28] from literature review, is mainly owing to production of ROS, such as superoxide anion (O^{2-}), singlet oxygen (O_2) and hydroxyl radical (OH^-) [29] but its nanoconjugates did not create a significant reduction in viability of cancer cells. It showed that the ROS generation of ZnO NPs functionalized with polymers was decreased, which shows the reduction of cytotoxicity. The reduction of cytotoxicity of conjugates (F.A-PEG-ZnO) is due to less interaction nano ZnO with cells and less release of Zinc ions from ZnO [30]

Secondly Zn^{2+} and ROS contributed to the cytotoxicity mechanism. The reduction of free Zn^{2+} was the major reason for the reduction of cytotoxicity in the ZnO NPs functionalized with polymers. But ROS generation was primarily responsible for the damage of cell [31].

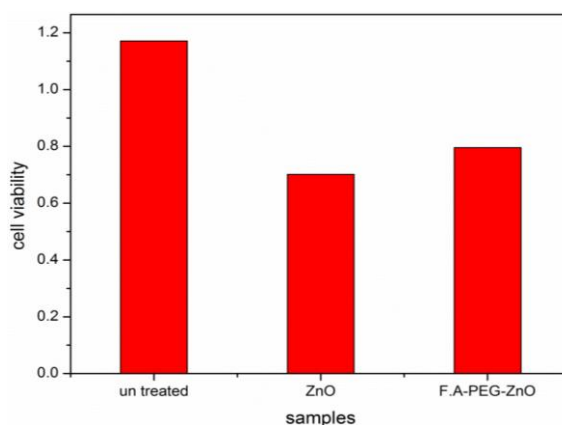


Fig. 4. Cytotoxicity of ZnO and F.A-PEG-ZnO.

4. Conclusion

ZnO NPs were fabricated by co-precipitation technique and were covered with polymeric layers (PEG + F.A). The covering which were examined through FT-IR analyses Cellular addition effectiveness ZnO NPs was greater than functionalized with polymers of PEG and F.A in HepG2 cells. Cell viability of individual ZnO NPs were less than functionalized by polymers. All these consequences point out that ROS generation of ZnO NPs decreases polymers coated, which leads to decrease its cytotoxicity. It concluded that Individual ZnO NPs have more cytotoxicity then functionalized with polymers.

References

- [1] Hahn, Y.-B., R. Ahmad, and N. Tripathy, Chemical Communications, 2012. 48(84): p. 10369; <https://doi.org/10.1039/c2cc34706g>
- [2] Rehman, N.-u.-., et al., Chemical Physics Letters, 2014. 609: p. 26; <https://doi.org/10.1016/j.cplett.2014.05.054>
- [3] Rehman, N.-u.-., et al., Applied Physics A, 2019. 125(8): p. 1; <https://doi.org/10.1007/s00339-019-2802-7>

- [4] Mamonova, I., et al., *Nanotechnologies in Russia*, 2015. 10(1): p. 128;
<https://doi.org/10.1134/S1995078015010139>
- [5] Yadavalli, T. and D. Shukla, *Nanomedicine: Nanotechnology, Biology and Medicine*, 2017. 13(1): p. 219; <https://doi.org/10.1016/j.nano.2016.08.016>
- [6] Singh, T.A., J. Das, and P.C. Sil, *Advances in Colloid and Interface Science*, 2020: p. 102317;
<https://doi.org/10.1016/j.cis.2020.102317>
- [7] Chen, J., et al., *Journal of Experimental Nanoscience*, 2008. 3(4): p. 321;
<https://doi.org/10.1080/17458080802235765>
- [8] Herman, D. and J.Y. Walz, *Langmuir*, 2015. 31(17): p. 4844;
<https://doi.org/10.1021/acs.langmuir.5b00745>
- [9] Stankus, D.P., et al., *Environmental science & technology*, 2011. 45(8): p. 3238;
<https://doi.org/10.1021/es102603p>
- [10] Loosli, F. and S. Stoll, *Environmental Science: Nano*, 2017. 4(1): p. 203;
<https://doi.org/10.1039/C6EN00339G>
- [11] Ajitha, B., et al., *RSC advances*, 2016. 6(42): p. 36171;
<https://doi.org/10.1039/C6RA03766F>
- [12] Granata, G., et al., *Journal of Nanoparticle Research*, 2016. 18(5): p. 133;
<https://doi.org/10.1007/s11051-016-3438-6>
- [13] Andreescu, S., et al., *Biomedical applications of metal oxide nanoparticles, in Fine particles in medicine and pharmacy*. 2012, Springer. p. 57; https://doi.org/10.1007/978-1-4614-0379-1_3
- [14] Kaweeterawat, C., et al., *Environmental science & technology*, 2015. 49(2): p. 1105;
<https://doi.org/10.1021/es504259s>
- [15] Elje, E., et al., *Nanomaterials*, 2020. 10(3): p. 545; <https://doi.org/10.3390/nano10030545>
- [16] Jevapatarakul, D., et al., *Biomedicine & Pharmacotherapy*, 2020. 130: p. 110552;
<https://doi.org/10.1016/j.biopha.2020.110552>
- [17] Khorsandi, L. and M. Farasat, *Environmental Science and Pollution Research*, 2020. 27(30): p. 38300; <https://doi.org/10.1007/s11356-020-09986-5>
- [18] Chen, P., et al., *Ecotoxicology and environmental safety*, 2019. 171: p. 337;
<https://doi.org/10.1016/j.ecoenv.2018.12.096>
- [19] Cai, X., et al., *Particle and fibre toxicology*, 2017. 14(1): p. 1;
<https://doi.org/10.1186/s12989-017-0193-5>
- [20] Nagvenkar, A.P., et al., *ACS omega*, 2019. 4(9): p. 13631;
<https://doi.org/10.1021/acsomega.9b00181>
- [21] Fiandra, L., et al., *NanoImpact*, 2020. 17: p. 100195;
<https://doi.org/10.1016/j.impact.2019.100195>
- [22] Saud Alarifi, D.A. and S. Alkahtani *International journal of nanomedicine*, 2015. 10: p. 3751;
<https://doi.org/10.2147/IJN.S82050>
- [23] Ahamed, M., et al., *Toxicology*, 2011. 283(2-3): p. 101;
<https://doi.org/10.1016/j.tox.2011.02.010>
- [24] Patterson, A., *Physical review*, 1939. 56(10): p. 978;
<https://doi.org/10.1103/PhysRev.56.978>
- [25] Dayakar, T., et al., *Materials Science and Engineering: C*, 2017. 75: p. 1472;
<https://doi.org/10.1016/j.msec.2017.02.032>
- [26] He, Y., et al., *Spectrochimica acta part A: molecular and biomolecular spectroscopy*, 2009. 72(4): p. 876; <https://doi.org/10.1016/j.saa.2008.12.021>
- [27] Venkatasubbu, G.D., et al., *Advanced Powder Technology*, 2013. 24(6): p. 947;
<https://doi.org/10.1016/j.appt.2013.01.008>
- [28] Yip, N., et al., *British journal of cancer*, 2011. 104(10): p. 1564;
<https://doi.org/10.1038/bjc.2011.126>
- [29] Sarwar, S., et al., *Nanomedicine: Nanotechnology, Biology and Medicine*, 2016. 12(6): p. 1499; <https://doi.org/10.1016/j.nano.2016.02.006>

- [30] Hsiao, I.-L. and Y.-J. Huang, Chemical research in toxicology, 2011. 24(3): p. 303;
<https://doi.org/10.1021/tx1001892>
- [31] Zhang, L., et al., Science of the total environment, 2019. 647: p. 313;
<https://doi.org/10.1016/j.scitotenv.2018.07.468>