Electronic Supplementary Material (ESI) for Physical Chemistry Chemical Physics.

Supporting Information

Cytotoxicity of ZnO nanoparticle under dark conditions via oxygen vacancy dependent reactive oxygen species generation

Aritra Biswas, Uddalok Kar and Nikhil R. Jana*

School of Materials Science, Indian Association for the Cultivation of Science,

2A & 2B Raja S. C. Mullick Road, Kolkata-700032, India

*Address for correspondence to camnrj@iacs.res.in

Experimental Section.

Materials. Methylthiazolyl diphenyl tetrazolium bromide (MTT) was purchased from Himedia, India. Zinc acetate dihydrate, oleic acid, poly(ethyleneglycol) methacrylate, 3-sulfopropyl methacrylate potassium salt, gallium nitrate hydrate, oleylamine, Igepal CO-520, propidium iodide (PI), ammonium persulfate, bis[2 (methyacryloyloxy)ethyl]phosphate, cyclohexane, tetramethyl ammonium hydroxide (5% methanol solution), dialysis tube (MWCO ~ 12,000-14,000 Da), N,N,N,N tetramethylethylenediamine, N-(3-aminopropyl) methacrylamide, Dulbecco's Modified Eagle Medium (DMEM), 2′, 7′-dichlorofluorescin diacetate (DCF-DA), nitro blue tetrazolium (NBT) and terephthalic acid were purchased from Sigma-Aldrich. Ammonia solution was obtained from Merck.

Instrumentation. Transmission electron microscopy (TEM) images were measured using ultra high-resolution field emission gun transmission electron microscope (UHRFEG-TEM, JEOL, JEM 2100 F). Wide-angle X-ray diffraction (XRD) was measured with Bruker D8 Advance powder diffractometer using Cu Kα (λ = 1.5406 Å) as the incident radiation. X-ray photoelectron spectroscopy (XPS) measurement was performed using Omicron (serial number: 0571) X-ray photoelectron spectrometer. Raman spectra were recorded by J-Y Horiba Confocal Triple Raman Spectrometer (Model: T64000) using a 532 nm Nd:YAG laser (Spectra Physics). Emission spectral measurement was carried out using Perkin Elmer Fluorescence Spectrometer LS 45. UV−visible absorption and emission spectra were recorded using Shimadzu UV-2550 UV−visible spectrophotometer and Synergy Mx Multi-Mode Microplate Reader, respectively. The hydrodynamic sizes along with ζ potentials of the aqueous samples were measured using NanoZS (Malvern) instrument. Olympus IX81 microscope was used to capture the differential interference contrast (DIC) and fluorescence (F) images of cells using Image-Pro Plus version 7.0 software.

Synthesis approach of ZnO, Ga doped ZnO and $ZnGa_2O_4$ nanocrystals. About 150 mg of zinc acetate dihydrate was loaded in a 25 mL of three neck round bottomed flask, and dissolved completely by adding minimum amount of methanol. Next, tetramethyl ammonium hydroxide (TMAH) was added dropwise to the above solution until complete precipitation. The precipitate thus obtained was washed with methanol to remove unreacted TMAH. After that precipitate was dispersed in 7 mL oleic acid-oleylamine mixture (volume ratio 3:4) and heated at 70-80 °C for 30 min at argon atmosphere. Next, the temperature was increased to 200 °C and stirred additionally for 2 h. As the temperature was increased gradually the reaction mixture becomes clear and colourless, and after 1-2 h of heating a white colloidal dispersion of the nanoparticle was obtained. For, gallium doping similar reaction condition was followed, only gallium nitrate was added in the beginning with zinc precursor.

Gallium oxide nanoparticle was also synthesized separately following similar reaction as mentioned above using 255 mg of gallium nitrate as precursor and heated at 200 °C for 1 h under stirring with argon atmosphere. $ZnGa_2O_4$ nanoparticle was synthesized using 109 mg of zinc acetate dihydrate and 255 mg of gallium nitrate in methanol solution and mixed hydroxides are precipitated using TMAH/ammonia solution. Next, unreacted ammonia solution was discarded and the reaction mixture was mixed with oleic acid-oleylamine mixture (volume ratio 3:4) and reacted at 80 °C for 1-2 h, followed by heating at 200 °C for 2 h. In order to characterize the nanoparticles produced, a part of the solution was collected and precipitated from the solution by adding acetone and the isolated precipitate was dissolved in chloroform. Nanoparticles were further purified via ethanol-based precipitation and chloroform/cyclohexane/toluene-based redispersion for two times. Finally, purified nanoparticles were dissolved in toluene/chloroform and used for characterization studies.

As synthesized nanoparticles were hydrophobic and thus non-dispersible in aqueous medium. Hydrophobic nanoparticles were converted to water dispersible nanoparticles following a

polyacrylate based polymer coating with zwitterionic surface charge. First, as synthesized nanoparticles were purified using acetone/ethanol-based precipitation and chloroform based redispersion. These steps were repeated twice in order to remove any free ligands in the system and finally purified nanoparticles were taken in 2 mL cyclohexane. 3-5 mg nanoparticles were dissolved in 1.5 mL cyclohexane followed by 0.5 mL Igepal to prepare Igepal-cyclohexane reverse micelle. Similarly, reverse micelle solution of acrylate monomers are prepared separately and mixed with nanoparticle solution to make a total volume of 10-12 mL. Three acrylate monomers were used such as 36 μL poly(ethylene glycol) methacrylate (for introducing polyethylene glycol), 12.5 mg 3-sulfopropyl methacrylate (for introducing anionic SO_3^-) and 9 mg N-(3-aminopropyl) methacrylamide (for introducing primary amine groups). Finally, 5 moles % bis[2-(methyacryloyloxy)ethyl] phosphate was used as a cross-linker. A small fraction of fluorescein O-methacrylate was used in selected case in order to introduce fluorescein on the polymer backbone for cellular imaging. The whole solution was taken in a 50 mL three necked round-bottom flask, mixed with 100 μL of N,N,N,N tetramethyl ethylenediamine base and degassed under argon atmosphere for 15 min. After that, aqueous solution of ammonium persulfate (4 mg dissolved in 100 μL water) was injected to the reaction mixture and reaction was continued for 30 min under stirring conditions. Next, ethanol was added to precipitate the nanoparticles and repeatedly washed with chloroform/ethanol. Finally, nanoparticles were dissolved in water and purified by dialysis against double distilled water using dialysis membrane (MWCO ~12,000-14,000 Da).

Calculation process for the atomic percentages of lattice Zn and lattice O.

Calculation process for the atomic percentages of lattice Zn and lattice O was followed from the reference 21, which are briefly described below:

Integral area of Zn (I_{Zn}) and integral area of O (I_O) should be obtained. Then, divide the integral area of Zn with the atomic sensitivity factor of Zn (S_{Zn}) and divide the integral area of O with the atomic sensitivity factor of O (S_O). Through normalizing the two data, the percent of Zn (C_{Zn}) and the percent of O (C_O) were obtained:

$C_{Zn} = (I_{Zn}/S_{Zn})/[(I_{Zn}/S_{Zn})+(I_O/S_O)]$

$C_O = (I_O/S_O)/[(I_{Zn}/S_{Zn})+(I_O/S_O)]$

After fitting the XPS spectra of Zn and O in ZnO, the percent of lattice Zn of the total Zn ($P_{L\text{-}Zn}$) and the percent of lattice O of the total O ($P_{L\text{-}O}$) are obtained. The atomic percentages of lattice Zn ($A_{L\text{-}Zn}$) and atomic percentages of lattice O ($A_{L\text{-}O}$) are then calculated as follows:

$A_{L\text{-}Zn} = P_{L\text{-}Zn} \times C_{Zn}$ and $A_{L\text{-}O} = P_{L\text{-}O} \times C_O$

Reactive oxygen species (ROS) detection under light/dark.

Reactive oxygen species (ROS) under dark condition was determined following 2′,7′-dichlorofluorescin diacetate (DCF-DA) analysis. Briefly, 200 μL of nanoparticle dispersion (1 mg/mL) was mixed with 2 mL aqueous solution containing 10 μL DCF-DA (10 mM) and stirred under complete dark condition for 0.25-7 days and followed using fluorescence spectroscopy. In order to estimate superoxide radical ($\bullet O_2$) and hydrogen peroxide (H_2O_2), nitro blue tetrazolium (NBT) test and iodometry analysis were used, respectively. Briefly, 200 μL of nanoparticle dispersion (1 mg/mL) was mixed with 2 mL aqueous solution containing 10 μL nitro blue tetrazolium (0.05 mM) and stirred under complete dark condition for 0.25-7 days and followed using UV-visible absorbance spectroscopy. Hydrogen peroxide (H_2O_2) generated by the nanoparticles was determined under similar condition by mixing 200 μL of nanoparticle dispersion (1 mg/mL) with 2 mL aqueous solution containing 0.5 mL of NaCl solution (200 g/L), 0.2 mL of HCl solution (3.6 vol %), 0.3 mL of KI solution (10 g/L), and 0.2 mL of soluble starch solution (10 g/L), successively and followed using the UV-visible absorption spectroscopy.

Terephthalic acid reacts with hydroxyl radical (•OH) and produce fluorescent 2-hydroxy-terephthalate. The reaction product is stable for hours and can be easily detected using a fluorimeter. Hydroxyl radical generated in presence of visible light (250 W Hg vapour lamp, wavelength: ≥380 nm, intensity: ~5 mW/cm^2) was monitored by mixing 200 μL of different nanoparticles (1 mg/mL) with 50 mL terephthalate (0.5 mM). Before visible light irradiation the reaction mixture was stirred for 30 min in the dark to establish adsorption-desorption equilibrium. At different time intervals a part of the solution was collected, nanoparticles were separated by centrifuge, and fluorescence of the supernatant containing 2-hydroxy-terephthalate was measured by exciting at 315 nm.

Investigation of nanoparticle-induced reactive oxygen species generation inside HeLa cells under dark. HeLa cells were cultured in a 24-well plate under 37 °C with 5 % CO_2 using DMEM media with 10 % heat activated fetal bovine serum (FBS) and 1 % penicillin streptomycin. After 24 h, cells were taken in fresh media and mixed with polyacrylate coated nanoparticle solution (final concentration 4-6 μg/mL). After 40-60 min of incubation cells were washed with PBS buffer solution (pH = 7.4) to remove unbound nanoparticles. Then cells were taken in fresh media and incubated under dark condition. For ROS detection, 10 μL of DCF-DA solution (10 mM) was added to the media and incubated for 10-15 min under 37 °C. Next, cells were washed with PBS (pH = 7.4) and fresh DMEM media was added. After that, the cells were imaged under blue excitation using fluorescence microscope. In order to determine the membrane damage, visible light irradiated cells were treated with PI staining solution (10 μg/mL), incubated in dark at 37 °C for 10 min, and washed cells were imaged under green excitation.

MTT Assay. HeLa cells were seeded into 24-well tissue culture plates in the presence of 500 μL of DMEM medium supplemented with 10 % fetal bovine serum and 1 % penicillin/streptomycin at 37 °C and 5% CO_2. After 24 h, cells were taken in fresh media and

then cells were incubated with polyacrylate coated doped nanoparticles for 60 min followed by 24 h incubation under dark. Next, cells were washed with PBS buffer, and 500 μL of fresh DMEM medium was added. Then, cells were incubated with 50 μL of MTT (5 mg/mL) solution for 4 h, violet formazan was dissolved in a DMF-water solution of sodium dodecyl sulfate, and absorbance of the solution was measured at 570 nm in a microplate reader. The relative cell viability was measured by assuming 100 % cell viability for control cells without nanoparticles.

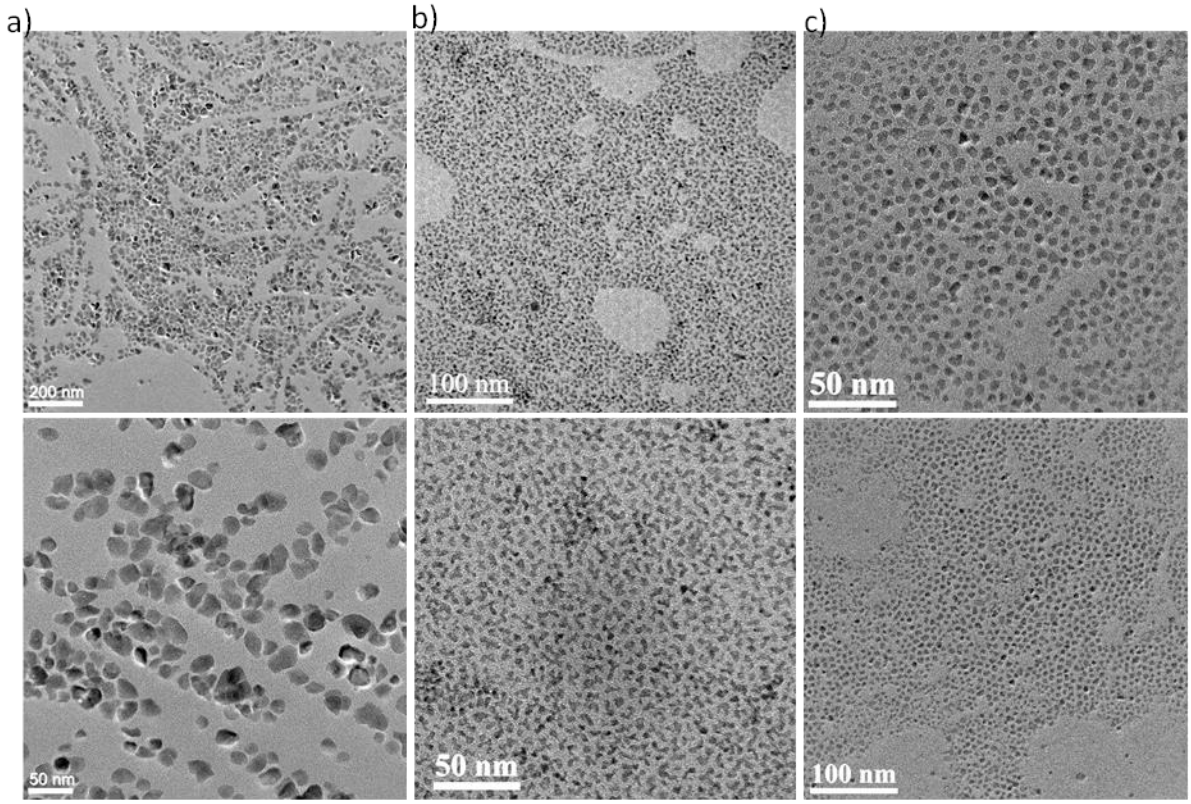


Figure S1. Additional transmission electron microscope (TEM) images of a) ZnO, b) Ga doped ZnO, and c) $ZnGa_2O_4$ nanoparticles at two different magnifications.

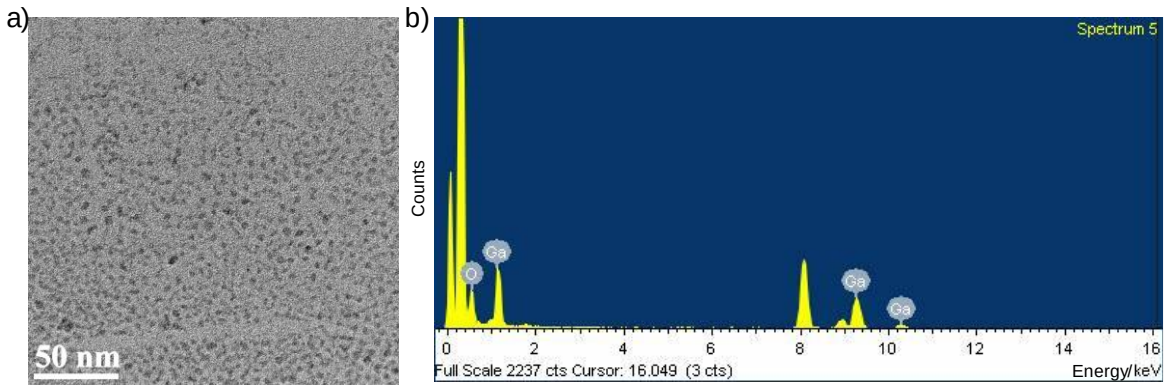


Figure S2. a) Transmission electron microscope (TEM) image and b) EDS spectrum of gallium oxide nanoparticle formed as intermediate of reaction after one hour of reaction.

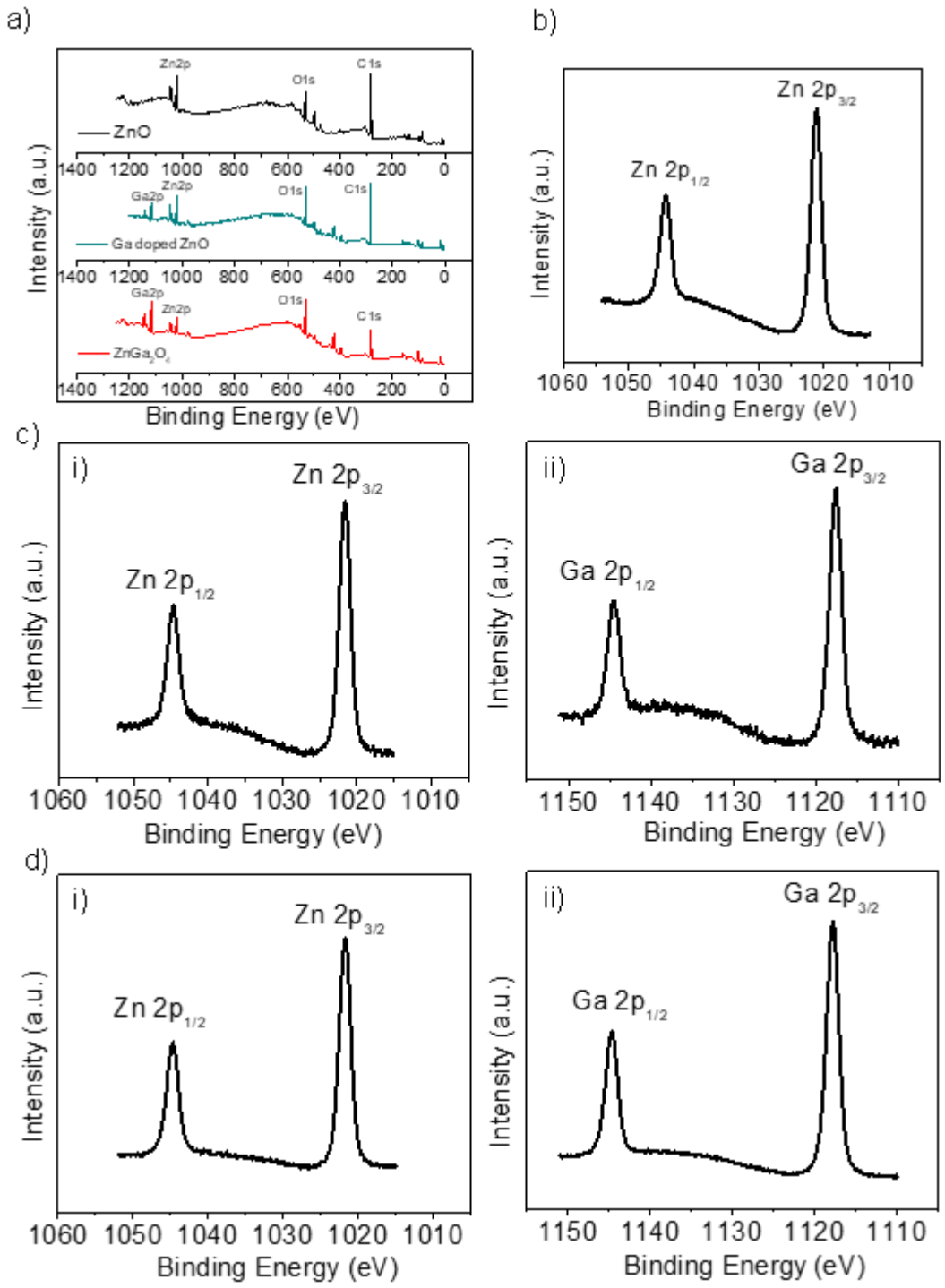
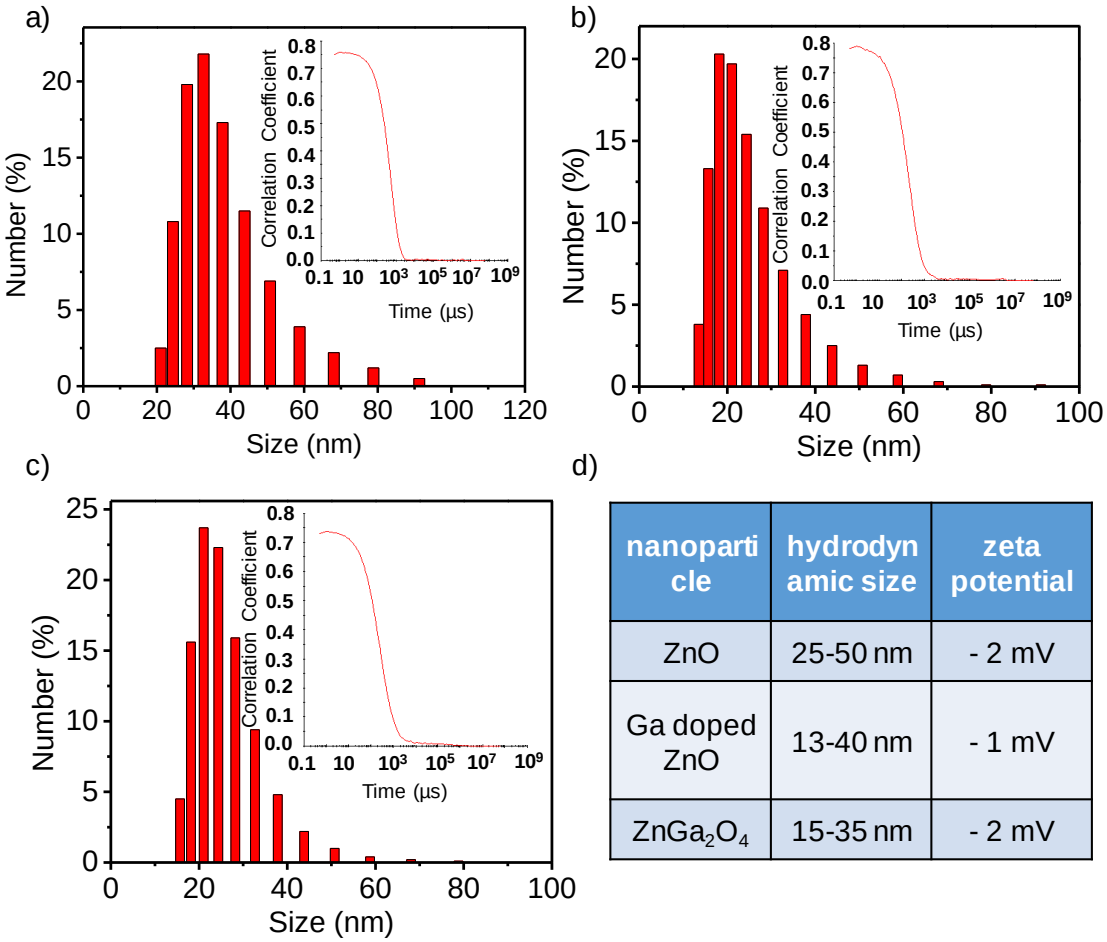


Figure S3. a) Low resolution XPS survey scan of 3 nanoparticles showing the presence of zinc, gallium and oxygen as major elements present in the nanoparticles. b-d) XPS high resolution spectra for Zn 2p for ZnO (b), Zn 2p (i) and Ga 2p (ii) in Ga doped ZnO (c) and Zn 2p (i) and Ga 2p (ii) in $ZnGa_2O_4$ (d) nanoparticles.



nanoparticle	hydrodynamic size	zeta potential
ZnO	25-50 nm	- 2 mV
Ga doped ZnO	13-40 nm	- 1 mV
$ZnGa_2O_4$	15-35 nm	- 2 mV

Figure S4. Hydrodynamic size of the polyacrylate coated ZnO (a), Ga doped ZnO (b) and $ZnGa_2O_4$ (c) nanoparticles, measured in phosphate buffer solution (pH=7.4) with correlation coefficient in inset. d) Summary of hydrodynamic size and surface charge (zeta potential) of three nanoparticles in phosphate buffer solution (pH=7.4).

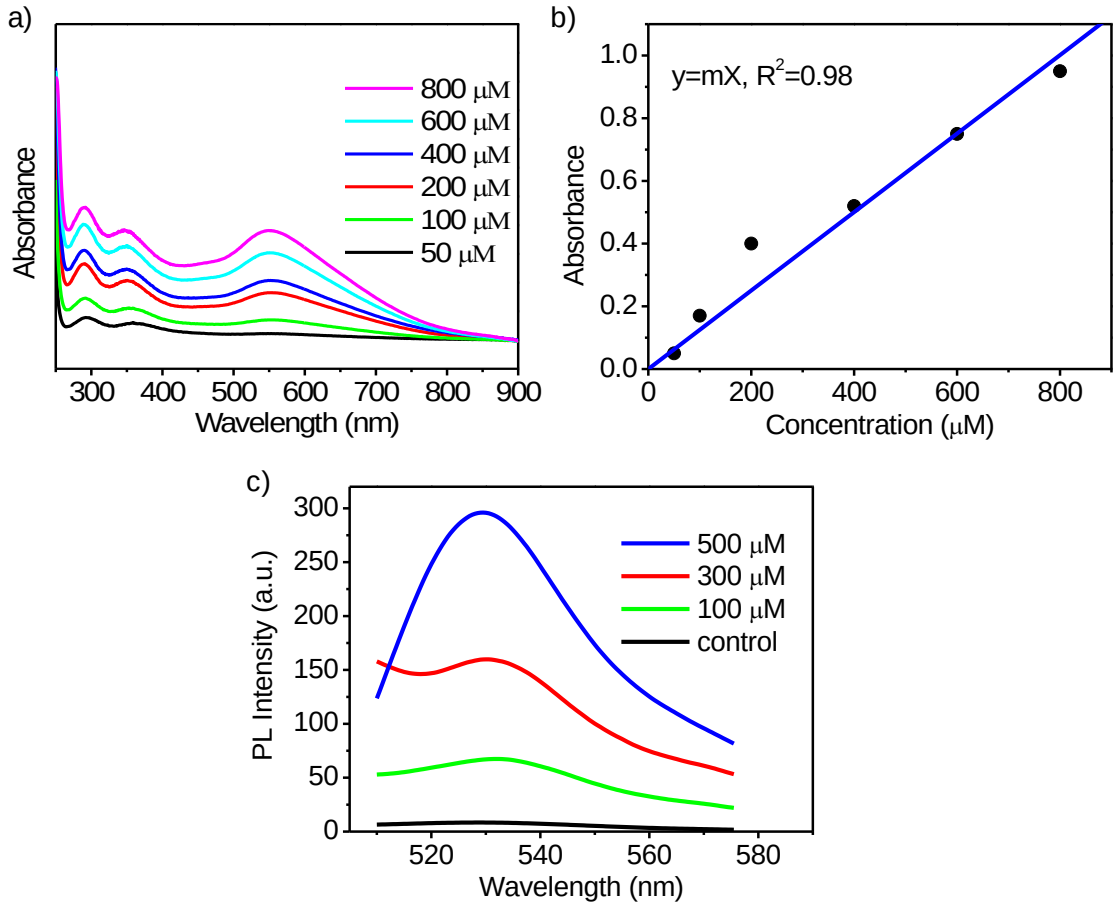


Figure S5. a) UV-visible absorption spectra and b) calibration curve of starch-iodine complex that are produced in presence of different concentration of hydrogen peroxide. c) DCF-DA emission spectra in presence of different concentration of hydrogen peroxide after 2h. The hydrogen peroxide concentrations adjusted so that they produce ROS similar to different ZnO nanoparticles as shown in Figure 4.

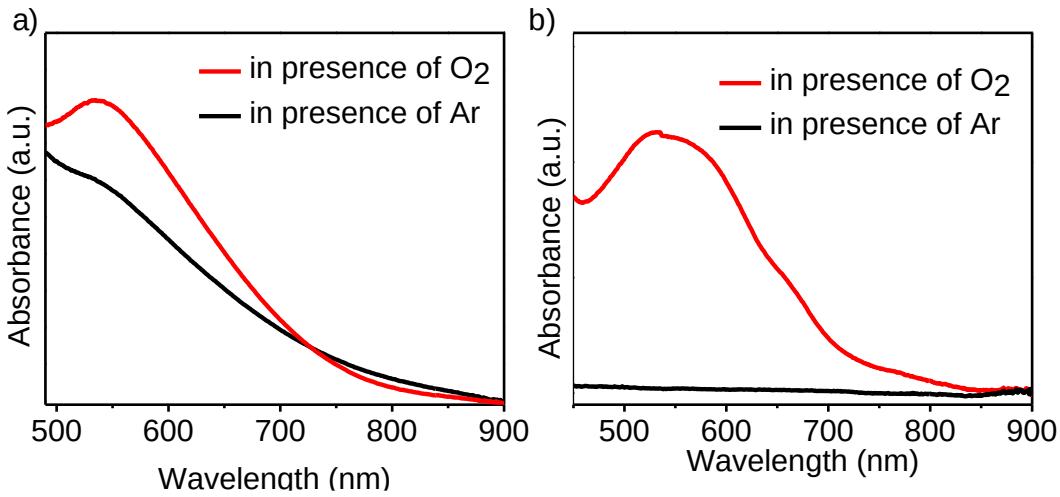


Figure S6. KI-starch-based estimation of generated hydrogen peroxide (a) and NBT-based estimation of generated superoxide ($\cdot O_2^-$, b) from ZnO nanoparticle in presence/absence of dissolved oxygen. Argon purging was used to remove dissolved oxygen from water. Results indicate significant decrease in ROS generation in absence of dissolved oxygen.

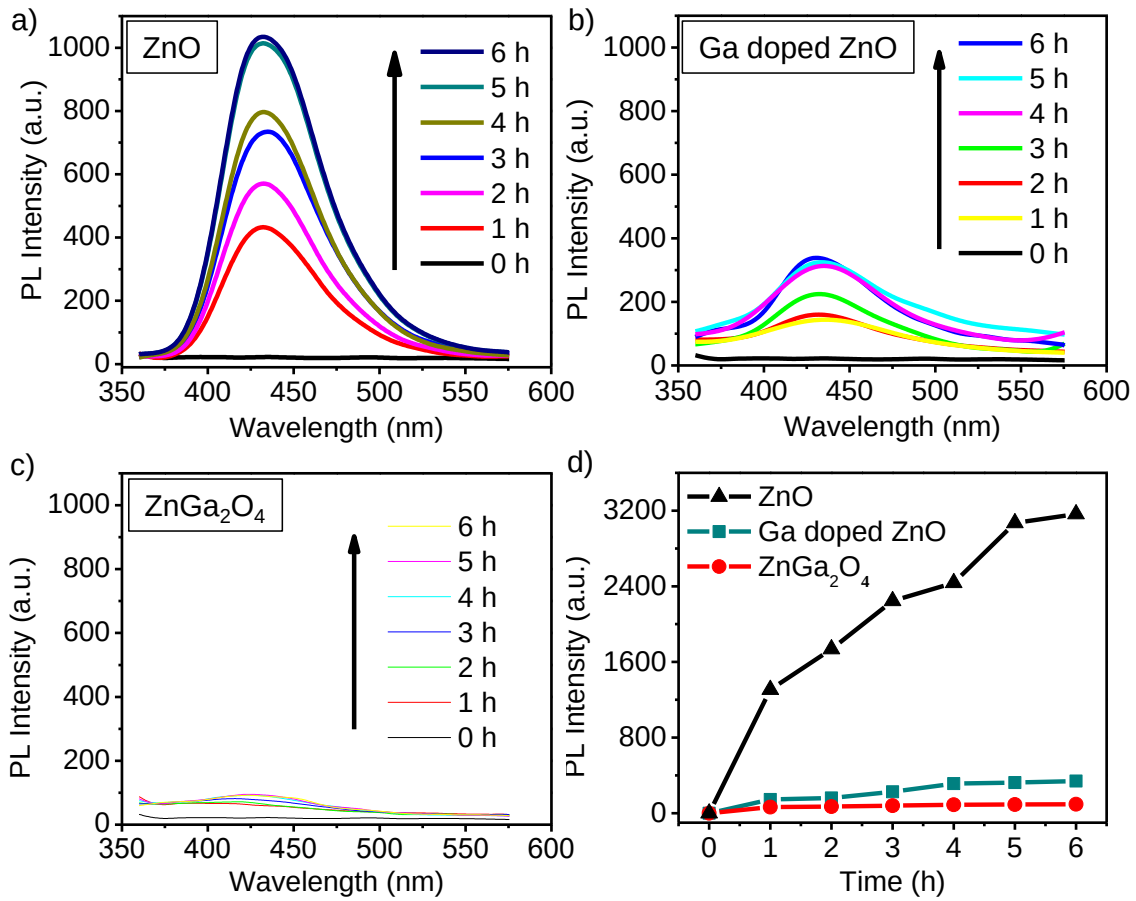


Figure S7. Evidence of hydroxyl radical generation by oxygen deficient ZnO nanoparticle under visible light exposure. Typically, colloidal solution of ZnO (a) or Ga doped ZnO (b) or $ZnGa_2O_4$ (c) is mixed separately with terephthalic acid and irradiated with visible light. Next, the generated hydroxyl radicals are detected as emission at 430 nm under excitation at 315 nm. d) Comparative formation of hydroxyl radicals from different nanoparticles, showing that hydroxyl radical generation decreases with increased Ga doping.

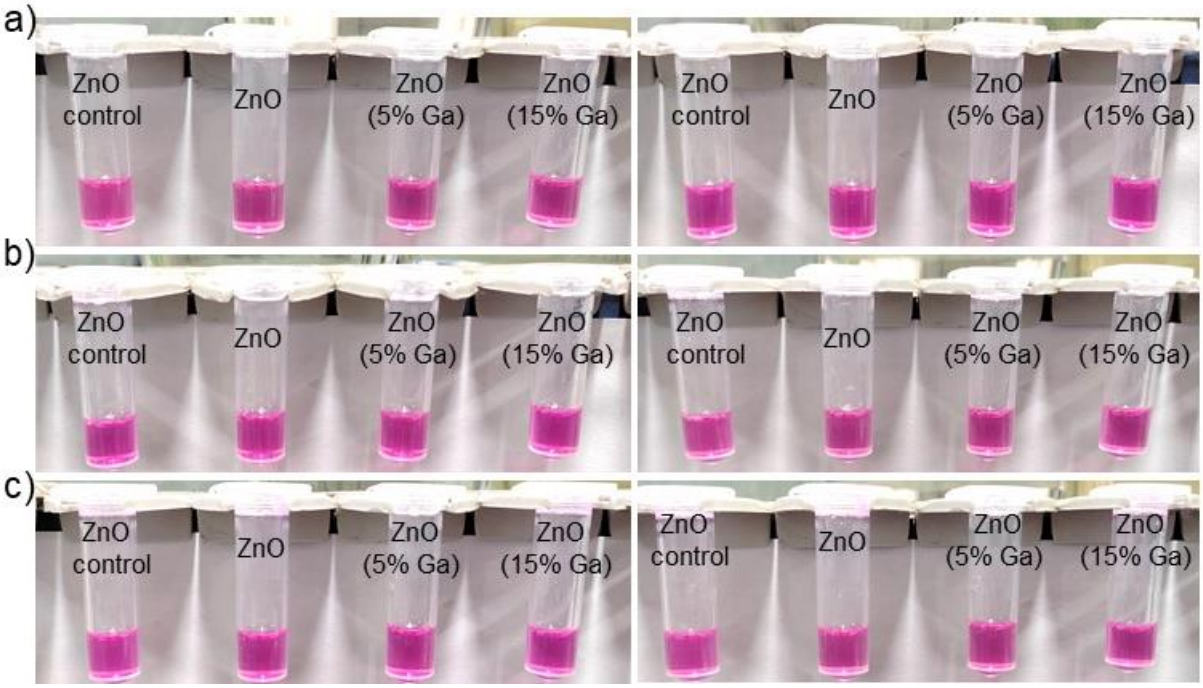


Figure S8. Colloidal stability of zinc oxide nanoparticles of different concentrations in cell culture media taken at different time interval. a) 0.1 mg/mL, b) 0.2 mg/mL and c) 0.4 mg/mL (left: 0 h, right: after 24 h) Results indicate the nanoparticles are stable in cell culture media without any precipitation.

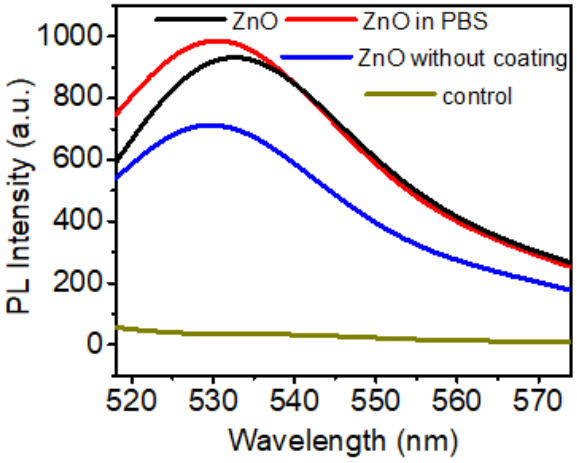


Figure S9. DCF-DA-based fluorescence spectral data of colloidal solution of polyacrylate coated ZnO nanoparticles in water (ZnO), same nanoparticle in phosphate buffer solution of pH 7.4 (PBS) and same nanoparticle without polyacrylate coating (control). Results show the more or less intact ROS generation property of ZnO nanoparticles for all samples. ROS generation slightly decreased for uncoated nanoparticles due to poor water dispersibility of hydrophobic nanoparticles.

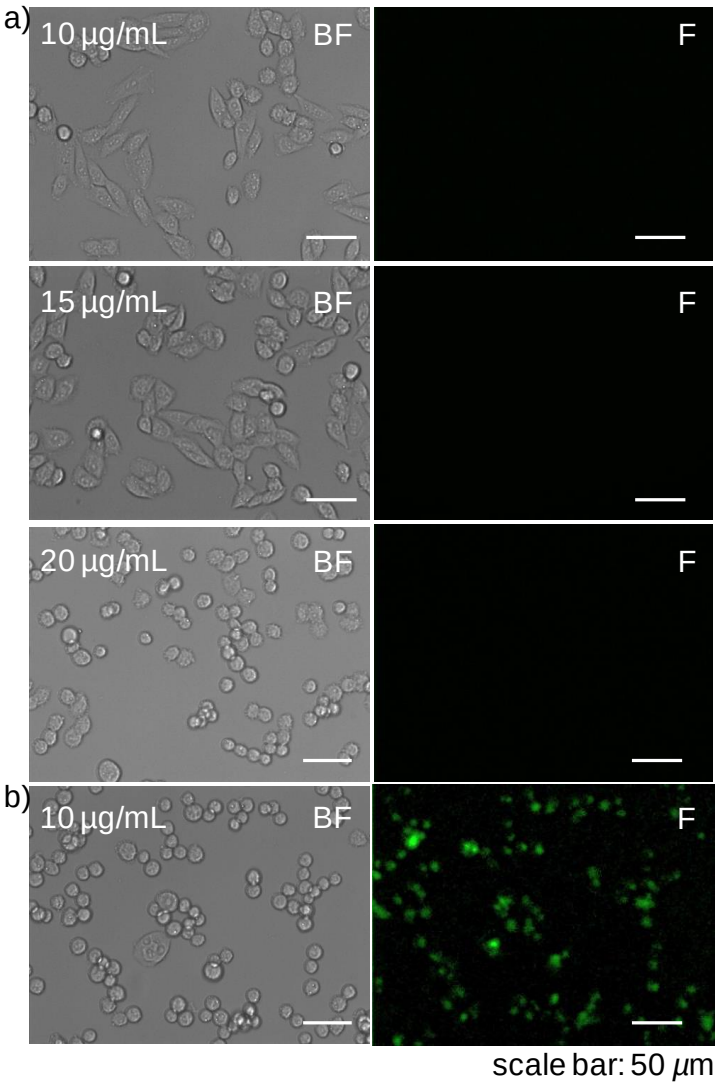


Figure S10. a) Cytotoxicity of CHO cells in presence of different concentrations of zinc acetate solution (10-20 µg/mL). Cells are incubated with zinc acetate solution for 40 min and then ROS is imaged using DCF-DA-based fluorescence microscopy. Washed cells are imaged under bright field (BF) or fluorescence (F) mode. Absence of green fluorescence (F, right panel) indicates insignificant ROS generation. Cells are found healthy as observed from the well-defined morphologies in presence of 10-15 µg/mL zinc ions. However, the cytotoxicity is observed for 20 µg/mL zinc ions with cell deformation, although no ROS is observed. b) Cytotoxicity of CHO cells in presence of ZnO nanoparticles after 40 min incubation in dark. The relatively lower nanoparticle concentration (6-10 µg/mL) produces significant intracellular ROS and cell deformation.

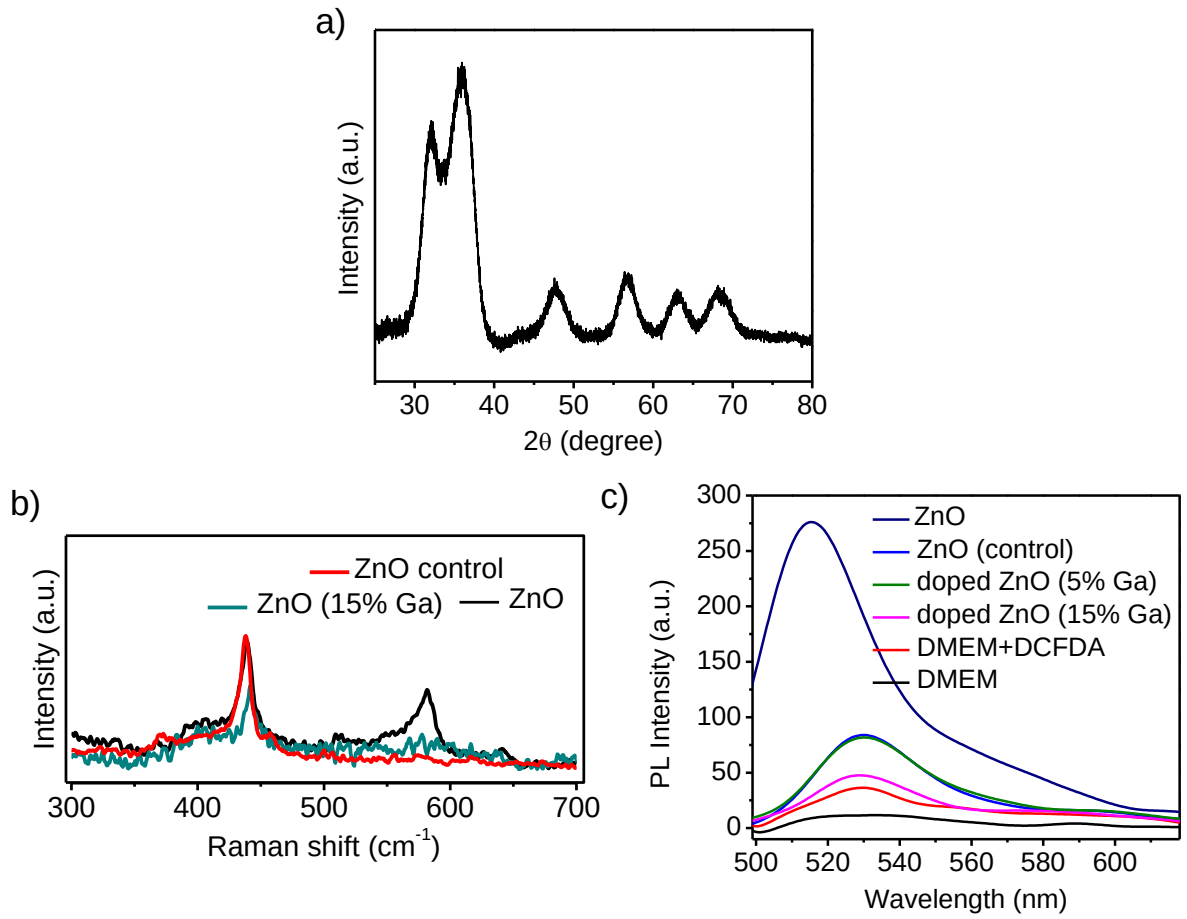


Figure S11. a) XRD pattern of control ZnO nanoparticles. b) Raman spectra of different nanoparticle showing the presence of oxygen vacancy associated broad band in the range of 500-600 cm^{-1} that decreases after Ga doping and almost insignificant in case of control zinc oxide nanoparticles. c) Comparison of overall ROS generation under dark by oxygen deficient ZnO nanoparticle and other nanoparticles/conditions, using DCF-DA probe. Same amounts of nanoparticles (1.5 mg/mL) are used in all these experiments. ROS generation is low for Ga doped ZnO nanoparticles and control zinc oxide nanoparticles.

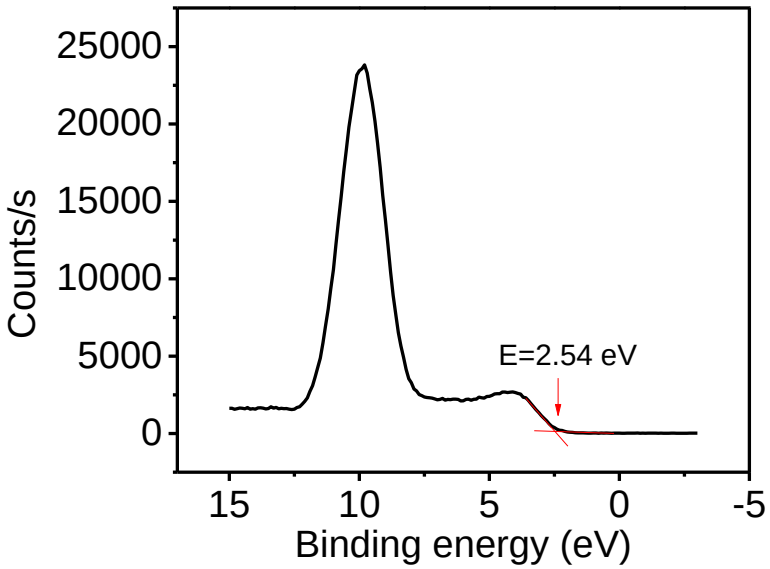


Figure S12. High resolution valence band XPS spectra of zinc oxide nanoparticle indicating the valence band maxima position.

Table S1. ICP-based analysis of zinc ion leaching in different solutions taken after 24 h.

solution	zinc ion concentration (µg/mL)
distilled water	0.003
phosphate buffer solution (PBS 7.4)	0.004
cell culture media	0.003