CHAPTER 5
The Mechanism of Action of PEG Functionalized Zinc Oxide against Different Breast Cancer Cell Lines

Abstract: Nanoparticles are increasingly recognized for their utility in biological applications including nanomedicine. The present study investigated the toxicity of zinc oxide (ZnO) nanoparticles toward highly proliferating breast cancer cell such as MCF-7, MDA-MB etc along with normal cells like peripheral blood mononuclear cells (PBMCs). In this investigation, we explored the mechanism by which ZnO nanoparticle induces apoptosis in different breast cancer cells. It was observed that Fas-FADD pathway was activated in breast cancer cells upon ZnO nanoparticles treatment. Fas-FADD is one of the crucial apoptosis pathways by which external ligands induce apoptosis. Fas-FADD activation ultimately resulted in cleavage of BID and alteration of mitochondrial transmembrane potential that leads to cytochrome-c release and activation of several caspases. Although this is the major pathway involved in cancer cell apoptosis, possibility of ROS mediated intrinsic apoptosis pathway could not be ruled out.

5.1. Introduction
Cancer happens due to the uncontrolled growth of abnormal cell which originates from the normal cells in the body. Normal cells generally multiply when the body needs them, and automatically die when the body doesn't need them. The process by which this programed cell death occurs is known as apoptosis. Cancer appears when the growth of cells in the body is out of control and cells division occurs too rapidly. It can also occur when cells forget how to die. The exact cause of cancer is still unknown; however, there are several factors which enhance the occurrence of cancer, such as exposure to benzene and other chemicals, environmental toxins, genetic problem, radiation, infection by some specific viruses, differences in diet, smoking habit and obesity. Cancer is reported as the second leading cause of death in the US and accounts for ~ 25% of total deaths. Even more threatening is the recent projection by the World Health Organization (WHO), which forecasted that within 2030 the total cancer population will become double compared to recent population. Despite the tremendous scientific advancement made in last decades to understand human body functioning at the molecular level, the therapeutic options for cancer treatment still remain very limited. Current anticancer chemotherapies mainly rely on alkylating agents, antimetabolites, biological agents and natural products,
which frequently fail to produce the desired response owing to the development of drug resistant cell line or the failure to differentiate effectively between cancerous and normal cells. This indiscriminate action of the present therapeutic regime recurrently leads to systemic toxicity and devastating adverse effects in normal body physiology, including bone-marrow malfunctioning, neurotoxicity and cardiomyopathy, which greatly limit the maximal allowable dose of the chemotherapeutic drugs. In addition, the rapid elimination or widespread dissemination of the anticancer drug across non-target tissues reduces their bioavailability and as an outcome large quantities of drug administration is required, which can further complicate problems related to nonspecific toxicity. Thus, there is an urgent need to develop new classes of anticancer drugs with new modes of action that can selectively target cancer cells while sparing healthy tissues. Recent investigations have shown that nanoparticles have high degree of selectivity towards cancer cell, which can be utilized for future anticancer drug development.

5.2. Nanoparticles and Cancer Treatment

The use of nanomaterials as drug carriers to enhance antitumor efficacy has been known for more than 30 years. The first investigation on the clinical potential of nano-drug carriers as liposomes occurred in the mid-1970s. Presently, the utilization of nanomaterials for the delivery of pharmaceutical and diagnostics agents remains an active field of research; recent progress has been reported by conjugating cell-specific ligands at the surface of nanoparticles, resulting in greater control of drug delivery at the target site, and by encapsulating drugs within the surface of nanoparticles to significantly improve the capacity of drug release. Several preclinical studies using nanoparticle-based therapies in oncology are underway, although some ideas have already been brought to the market (Table 1). The FDA-approved Abraxane® (Abraxis BioScience, AstraZeneca, London, England), an albumin-paclitaxel (Taxol®) nanoparticle treatment for metastatic breast cancer, has shown a promising overall response rate of 33%, compared with 19% for taxol alone in a randomized trial of 454 patients. Overall, the side effects were also negligible for nano-drug conjugate, even though it delivers a 50% higher dose of the active taxol at the target site compared to the conventional formulation. Another impressive example of nano drug conjugate is Myocet® (Cephalon, Frazer, PA), a liposomal...
formulation of doxorubicin that has significantly enhanced the therapeutic index compared to the conventional doxorubicin\textsuperscript{13} (Table 1).

<table>
<thead>
<tr>
<th>Product</th>
<th>Type of nanomaterial</th>
<th>Indication</th>
<th>Company</th>
<th>Phase</th>
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<td>Phase I</td>
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Table 1. Nanoparticle based cancer therapeutics in the clinical development pipeline.\textsuperscript{1}

Therapeutic index is the ratio of the amount of a pharmaceutical that causes the desired therapeutic effect to that which causes unwanted cell death. In addition to nano-drug carriers, interest is growing on the ability of certain nano-materials including metal oxides to mediate anticancer effects on their own. One approach involves the successful utilization of TiO\textsubscript{2} metal oxide nanoparticles against cancer cells in presence UV irradiation.\textsuperscript{14,15} Report shows that HeLa cells were completely killed when TiO\textsubscript{2} was treated in combination with UV irradiation, and tumor growth was arrested up to 30 days; interestingly, no killing of cancer cell was observed in the absence of TiO\textsubscript{2} nanoparticles and UV light. A major limitation of this photodynamic nanomedicine-based approach is the inability of UV light to penetrate > 1 mm through skin unless fiber optics or surgery is used in conjunction, although this treatment found effectiveness against several types of skin cancer. Nanomedicine-based hyperthermia is another promising field of anticancer therapy. Infusing a tumor with magnetic or metal nanoparticles and then exposing the patient to an alternating magnetic field or microwave radiation normally produces heat that warms areas immediately adjacent to the nanoparticles.\textsuperscript{16,17} When sufficient supernormal temperatures are achieved, the tumor cells are killed without harming surrounding healthy tissue. Both photodynamic and hyperthermic nanoparticle-based
cancer approaches share the challenge of preferentially accumulating at tumor sites, unless targeting strategies are also used. In addition to the above applications, metal oxide (such as zinc oxide) nanoparticles are gaining popularity for developing new generation of anticancer therapeutics.

5.3. Apoptosis and Cancer

Nanoparticle-induced cyto-toxicity can be broadly classified into two types i) Apoptosis, and ii) Necrosis. Apoptosis refers to programmed cell death in response to various intrinsic or extrinsic death signals. On the other hand, necrosis means the premature death of cell or living tissue due to acute injury and it is mostly caused by factors external to the cells. Apoptosis is controlled by cascade of cellular signaling events which may originate from intracellular or extracellular factors. There are certain hallmarks of apoptosis including activation of caspases (serine proteases), loss of mitochondrial transmembrane potential with release of cytochrome c (Cyt-c), extensive degradation of genomic DNA and formation apoptotic body. There are mainly two mechanisms by which cell commits apoptosis.

1. One generated by signal arising within the cell.
2. Another triggered by death activator binding to the receptor of the cell surface

The extrinsic or death receptor pathway is mainly constituted by Fas receptor (integral membrane receptor) and their receptor domain is always exposed to outer surface of the cell. Binding of the complementary death activator (FasL) transmits a signal to the cytoplasm that triggers cascade of caspase activation leading to phagocytosis of the cell (Figure 1). Defective apoptosis (programmed cell death) represents a major causative factor in the development and progression of cancer. The ability of tumor cells to evade engagement of apoptosis can play a significant role in their resistance to conventional therapeutic regimen.
Understanding of the complexities of apoptosis and the mechanisms evolved by tumor cells to resist engagement of cell death has focused research effort into the development of strategies designed to selectively induce apoptosis in cancer cells.

5.4. Zinc oxide Nanoparticle, Apoptosis and Prevention of Cancer

ZnO nanoparticles have multiple properties that are useful for biomedical applications, such as favorable band gap, electrostatic charge, surface chemistry and potentiating of redox-cycling cascades. Reduction of ZnO to the nanoscale has some unique consequences like toxicological ramifications, and the generation of reactive oxidative species (ROS), which can be exploited in combination with cancer-specific chemotherapeutic agent for developing new generation of therapeutics. A variety of metal oxide nanoparticles have shown successful application as vehicles for drug delivery, targeted gene delivery and tumor imaging. The use of metal oxide nanoparticle, particularly ZnO, in these applications is being explored, with some promise in areas of drug carrier and targeted gene delivery. Recently several studies have shown an increase
in cytotoxicity with nano ZnO compared to micrometer sized ZnO for different types of cancer, including colon, breast, bone, glioma, leukemias and lymphomas. However, majority of these studies do not compare cancer cell cytotoxicity with relevant non-immortalized cell types. The most compelling evidence of nano ZnO selective toxicity arises from controlled studies comparing nanoparticle susceptibility of cancerous cells with primary non-immortalized cells of identical activity. This investigation further revealed that cancerous cells of lymphocytic lineage were ~ 32 times more susceptible to ZnO nanoparticles mediated cytotoxicity as compared to their bulk counterparts. This high degree of specificity exceeds the ex vivo therapeutic indices normally reported for commonly used chemotherapeutic drugs, such as doxorubicin and carboplatin against a number of leukemias, lymphomas and solid tumors using similar biological assays. The preferential cytotoxicity was found to be dependent on the proliferation status of cells, with rapidly dividing cells being the most common target. Based on a increasing number of evidence, ROS production is proposed to be a major killing mechanism of ZnO nanoparticles, leading to cell death by means of an apoptotic mechanism. Literature evidences further demonstrated that co-administration of ZnO nanoparticles and the chemotherapeutic drug daunorubicin resulted in synergistic cytotoxic effects on leukemic cancer cells, which was enhanced further by UV irradiation. Despite these reports the selective cyto-toxicity of nano ZnO is still under intense debate as there is no clear cut mechanism available in literature to verify the above claim. This is a very promising area of research and future efforts in this area are expected to explore the mechanism of action along with direct drug conjugation or encapsulation within the ZnO nano-crystal to improve further the anticancer efficacy.

5.5. Motivation of the Work

We have initiated this investigation with some specific objectives. 1) Does ZnO NP show any selective cyto-toxicity against cancer cells? 2) Whether polyethelene glycol (PEG) capping (surface functionalization) has any role in reducing toxicity of ZnO towards normal cells? 3) How does ZnO NP induce apoptosis in cancer cells? 4) How efficient is this treatment compared to the presently available therapeutics? Our finding clearly shows that PEG conjugated ZnO can be effectively used against breast cancer cell and the virtue of this material lies in its limited side effect towards healthy normal cell line. For
our investigation we have purposefully selected different breast cancer cell lines as breast cancer is spreading at an alarming rate and the present treatment regimen is insufficient to cure the disease completely. The treatment is also very expensive and out of reach of common people.

5.6. Materials and Methods

5.6.1. Materials. Zinc acetate dihydrate \([\text{Zn}(\text{CH}_3\text{COO})_2\cdot2\text{H}_2\text{O}]\), lithium hydroxide monohydrate \((\text{LiOH}\cdot\text{H}_2\text{O})\), ethanol, \(n\)-hexane, polyethylene glycol (PEG), and Fluorescein isothiocyanate were purchased from Sigma-Aldrich and used as received. Fetal bovine serum (FBS), Dulbecco’s modified Eagle’s medium (DMEM), streptomycin, penicillin, tetracycline, insulin, L-glutamine, sodium pyruvate were obtained from Gibco BRL (Gaithersburg, MD, USA). 4P,6P-diamidino-2-phenylindole (DAPI), T4 polynucleotide kinase and general reagents were purchased from Sigma (St. Louis, MO, USA). Polyclonal antibodies, horseradish peroxidase conjugated goat anti-mouse and goat anti-rabbit antibodies were obtained from Pharmingen (San Diego, CA, USA). Wild-type anti-p53 antibody (monoclonal) was obtained from Oncogene Science (Cambridge, MA, USA). All other reagents used in these experiments were of analytical grade and purchased mostly from local vendor, double distilled water was used throughout the experiments.

5.6.2. Synthesis of ZnO Quantum Dots (QDs). The acetate adsorbed ZnO QDs (i.e., ZnO-Ac QDs) were synthesized by modified sol–gel route using zinc acetate dihydrate \([\text{Zn}(\text{CH}_3\text{COO})_2\cdot2\text{H}_2\text{O}]\), lithium hydroxide monohydrate \((\text{LiOH}\cdot\text{H}_2\text{O})\), ethanol and \(n\)-hexane. All reagents used were of analytical grade. 10 mM \([\text{Zn}(\text{CH}_3\text{COO})_2\cdot2\text{H}_2\text{O}]\) was refluxed in ethyl alcohol (EtOH) for 20 min to obtain clear solution and allowed to cool at room temperature. 20 mM LiOH·H₂O was sonicated in EtOH and added dropwise to zinc acetate solution with continuous stirring. The ZnO QD was precipitated using \(n\)-hexane and centrifuged. The ZnO QD powder was obtained by washing the precipitate with ethanol and dried at 60 °C. The synthesized ZnO surfaces were modified with several capping agents for different biological application.

5.6.3. Polyethylene Glycol (PEG) Capping. 2 ml of freshly prepared ethanolic solution containing ZnO QDs was diluted in 8 ml of ethanol. A separate solution of 50 mM PEG (MW 600) was prepared by stirring at 45 °C until the solution became clear; it was then...
cooled down to room temperature. To previously prepared ZnO solution, a portion of 300 µl of 50 mM aqueous PEG solution was added with vigorous stirring. The precipitate was collected by centrifuging. PEG capped ZnO NP (ZnO-PEG in short) were re-dispersed in the solvent of choice, e.g. water, ethanol etc.

5.6.4. Dye Fluorescein Isothiocyanate (FITC) Capping. A solution (I) containing 1 mg of FITC and 2 µl of (3-aminopropyl) triethoxysilane (APTES) was prepared in 500 µl of DMSO. The mixer was stirred on magnetic stirrer for 4-5 h. Centrifuged ZnO QDs were dispersed in 10 ml of diethylene glycol (DEG) under stirring. 200 µl of tetraethyl orthosilicate (TEOS) and 250 µl of APTES were added to DEG dispersed ZnO solution and stirred. After 5 min of mixing, the FITC-APTES solution (I) was added to this ZnO solution under stirring. The mixer was kept stirring overnight for efficient binding.

5.6.5. Nanoparticle Characterization

5.6.5.1. Electron Microscopy. The particle size and dispersity of the prepared nanoparticles were studied using transmission electron microscope (TEM). TEM grids were prepared by placing 10 µl of the diluted and well sonicated sample solutions on a carbon-coated copper grid and dried completely in dust free atmosphere. The bright field electron micrographs of the samples were recorded on JEM-2010 (device: Orius SC1000) at the accelerating voltage of 200 kV.

5.6.5.2. Atomic Force Microscopy. To determine the morphology of PEG-functionalized ZnO NPs on a silicon wafer surface, deposited by spin casting, the samples were analyzed ex situ by Atomic Force Microscopy (AFM). AFM characterization was carried out using a Digital Instruments Nanoscope III. AFM measurements were performed in tapping mode using a Si$_3$N$_4$ tip with resonance frequency of 100 kHz and spring constant being 0.6 N m$^{-1}$ to obtain surface topography of deposited ZnO-PEG NPs. The film was air dried in dust free environment before measurement.

5.6.5.3. FTIR Spectroscopy. FTIR technique was used to determine the binding of PEG to ZnO. FTIR scanning was performed in the transmission mode with constant nitrogen purging using Perkin-Elmer spectrometer equipped with a DTGS KBr detector and a KBr beam splitter with constant nitrogen purging. IR grade KBr was used as scanning matrix. 1-2 mg of fine sample powder and 90-100 mg of KBr powder were mixed and dried completely, then transferred to 13 mm die to make a nearly transparent and homogeneous
pallet. All spectra were taken at 4 cm⁻¹ resolution, averaged over 20 scans in the range 400 to 4000 cm⁻¹.

5.6.6. Cell Culture. Variety of human breast cancer cell lines including mutant p53, i.e. MDA-MB-231, MCF-7, T47D, HBL-100 and MDA-MB-468 were obtained from the national centre for cell science, India. Cells were routinely cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, insulin (0.1 U/ml), L-glutamine (2 mM), sodium pyruvate (100 µg/ml), non-essential amino acids (100 µM), streptomycin (100 µg/ml), penicillin (50 U/ml) and tetracycline (1 µg/ml) (Sigma, St. Louis, MO) at 37°C in a humidified incubator containing 5% CO₂. Peripheral blood mononuclear cells were collected from normal human volunteer and were maintained in medium consisted of RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 10 mM HEPES buffer, 2 mM L-glutamine, 50 µg of gentamicin/ml, 100 U of penicillin/ml, 100 µg of streptomycin/ml, and 0.25 µg of amphotericinB/ml. Cells were allowed to reach confluence before use. Viable cell numbers were determined by trypan blue exclusion assay.

5.6.7. Treatment of Cell. Cells were treated with different concentration of ZnO-PEG for different time points to select the optimum dose and the time required for killing. However, the doses beyond 30 µg/ml are found to be toxic for healthy human cells, restricting us to use 25 µg/ml dose for further studies. To understand the sequence of events leading to apoptosis, breast cancer cells were pretreated for 3 h with 50 µM each of the specific caspase-8 (z-IETD-FMK), caspase-9 (z-LEHD-FMK), broad-based pan-caspase (z-VAD-FMK) (Calbiochem, San Diego, CA) or for 1 h with mitochondrial pore inhibitor cyclosporin A (25 µM; Merck, Germany Whitehouse Station, NJ) prior to incubation with ZnO-PEG. To asses the involvement of Fas ligand (FasL) in apoptosis, MDA-MB-231 cells were pretreated with neutralizing anti-FasL antibody (BD Pharmingen, San Jose, CA)

5.6.8. Flow Cytometry. The nature of cell death was further determined using flow cytometry. The cells were double stained with fluorescein isothiocyanate (FITC)-conjugated annexin-V and propidium iodide (PI) or 7-Aminoactinomycin D (7-AAD) using an apoptosis kit (Invitrogen). Flow cytometry was performed using a FACS Calibur (FACSCalibur; Becton Dickinson) equipped with cell quest pro software (Becton...
Dickinson). The data were averaged over three identical experiments.\textsuperscript{20,27} For measurement of MTP loss, cells were loaded with potential-sensitive dye 3,3'-dihexyloxycarbocyanine iodide (DiOC\textsubscript{6}, Merck) and fluorescence of retained DiOC\textsubscript{6} was determined flow cytometrically using logarithmic amplification by CellQuest software (Becton Dickinson, San Jose, CA).\textsuperscript{28} To determine the surface expression of Fas, live cells were stained with FITC-conjugated anti-Fas antibody (BD Bioscience, San Jose, CA).

\textbf{5.6.9. Fluorescence Imaging.} For the identification of nuclear fragmentation and nanoparticle internalization cells were grown on cover slips. Cells were fixed with chilled methanol and stained with 4',6-diamidino-2-phenylindole (Molecular Probes, Eugene). The morphology of the cell nuclei was visualized using a fluorescence microscope (Leica microscope fitted with epifluorescence illuminator through a $\times60$ aperture oil immersion lens). To perform nanoparticle internalization assay, cells were first incubated with FITC-tagged nanoparticles for three hours, after these brief time of incubation cells were subjected to fixation and followed by DAPI staining. The NP localization inside the bacterial cell was visualized using a confocal microscope. To assay MTP (Mitochondrial Transmembrane Potential) loss, unfixed cells were loaded with potential-sensitive dye DiOC\textsubscript{6} and the fluorescence of retained DiOC\textsubscript{6} was visualized under microscope. To visualize cell surface Fas expression or Cyt-c release from mitochondria cells were primarily fixed with $\beta$-formaldehyde and permeabilized with Triton X-100 and then stained with anti-Fas/anti-Cyt-c antibody (Santa Cruz) followed by Alex Flour 546/488-conjugated secondary antibody and visualized with confocal microscope (Carl Zeiss, Thornwood, NY).

\textbf{5.6.10. RT-PCR and Western Blotting.} Two micrograms of total RNA, extracted with TRIzol reagent, were reverse transcribed and then subjected to PCR with enzymes and reagents of the RTplusPCR system (Eppendorf, Hamburg, Germany) using GeneAmp PCR system 2720 (Applied Biosystems, Foster City, CA). Primers for Fas and glyceraldehyde 3-phosphate dehydrogenase (internal standard) were 5'-CAAGGGATTGGAATTGAGGA-3' and 5'-GACAAAGCCACCCCAAGTTA-3'; and 5'-CAGAACATCATCCTGCCCTCT-3' and 5'-GCTTGACAAAGTGGTCGTTGAG-3' respectively. For western anlysis cell lysates were prepared in lysis buffer [20 mM Tris-
HC1 (pH 7.4), 100 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, and 1 mM EGTA containing protease inhibitors. Mitochondrial and cytosolic fractions were prepared according to Lahiry et al. A total of 50 μg of protein was separated by SDS-PAGE and transferred to nitrocellulose filter paper for Western blotting using required antibodies like, anti-caspases, anti-FasL, anti-FADD, anti-tBid, anti-Cyt-c (C-20), anti- MnSOD (N-20) from Santa Cruz. For co-immunoprecipitation, to determine the direct interaction between, FasL and FADD along with IgG and FADD the proteins immunopurified from cells lysates (200 μg protein) with anti-FasL antibody and protein A-Sepharose beads. The immuno-precipitates were Western blotted with anti-FADD antibody. The blots were developed with NBT/BCIP (1:1). In parallel experiment equivalent amount of protein was Western blotted with anti-actin antibody (C-2; Santa Cruz) to confirm equal protein loading.

5.6.1. Toxicity Screening. Peripheral blood mononuclear cell (PBMC) has been used here to check the toxicity of PEG modified ZnO nanoparticle. In brief, PBMC were isolated from whole blood by centrifugation through Ficoll-Hypaque solution and were seeded equally in 6 well tissue culture grade plates containing RPMI-1640 media supplemented with 10% FBS (foetal bovine serum, Pan Biotech) and incubated at 37 °C in a 5% CO₂ incubator. PBMC cells were further treated with nano-particles at different concentrations ranging from 5-100 μg/ml for 3 h followed by double staining with fluorescein isothiocyanate (FITC)-conjugated annexin-V and propidium iodide (PI) or 7-aminoactinomycin D (7-AAD) using an apoptosis kit (Invitrogen). % of apoptotic cells were analyzed by flow cytometry using a FACS Calibur (FACSCalibur; Becton Dickinson) equipped with cell quest pro software (Becton Dickinson). The data were averaged over three identical experiments.

5.7. Results

5.7.1. Characterization of Nanoparticle. The ZnO NPs were prepared according to the modified sol-gel route and subsequently modified with PEG. PEG functionalized ZnO NPs were characterized using high resolution transmission electron microscopy (HRTEM), atomic force microscopy (AFM) and Fourier transform infrared spectroscopy (FTIR). The average size of ZnO-NP was found to be ~7 nm (Figure 2a), which increased to 150 nm on PEG capping as revealed by high resolution AFM image (Figure 2b). The
surface morphology measurements suggest that the spin casting increases the grain size of ZnO-PEG at silicon surface as compared to the dispersed small particles in solution. The presence of PEG on the surface of ZnO was further confirmed by FTIR (Figure 2c). The spectrum of ZnO-PEG showed a distinct peak around 578 cm\(^{-1}\) representing Zn-O metal oxide bond. Further, a broad and strong peak in 3200-3500 cm\(^{-1}\) is attributed to the absorption of O-H group in ZnO-PEG nanoparticles. The peak at 1384 cm\(^{-1}\) is assigned to the symmetric C-H bending together with C-O-H bending vibration mode of PEG molecule.

5.7.2. Toxicity of Nanoparticle. Toxicity of nanoparticle is a controversial issue and should be addressed before initiating any experiment. The cell line of peripheral blood mononuclear cell (PBMC) cell line has been used here to check the toxicity of ZnO-PEG and normal ZnO. ZnO-PEG and ZnO mediated toxicity was calculated from the rate of apoptosis using standard fluorescence activated cell sorter (FACS) based assay. Both

![Figure 2. (a) TEM image of ZnO-PEG. (b) AFM image of PEG-functionalized ZnO NPs in 2D. (c) FTIR spectrum of ZnO-PEG in the transmittance mode.](image-url)
ZnO and ZnO-PEG demonstrated a dose dependent cytotoxicity against PBMC; however, ZnO shows several fold higher toxicity towards PBMC compared to ZnO-PEG. It was found that ZnO concentration 25 µg/ml is sufficient to cause apoptosis in more than 20% of PBMC cells; in contrary the same concentration of ZnO-PEG induces apoptosis in only 5% of PBMC cells (Figure 3a). The value remains much below the toxicity threshold of any anticancer pharmaceutics. The reduced toxicity of ZnO-PEG is attributed to biocompatible nature of PEG. If not specifically mentioned, further experiments were carried out with 25 µg/ml of ZnO-PEG found to be toxicologically safe.

5.7.3. Cytotoxicity of PEG-modified ZnO in Different Cancer Cell Lines. To examine the cytotoxicity of PEG-capped ZnO against cancer cell, we assessed the viability of different breast cancer cell lines (MCF-7, HBL-100, T47D) using fluorescence-activated cell sorter (FACS). We have observed a time dependent cytotoxicity of ZnO-PEG against all the cell lines, and the maximum effect was observed at 48 h post induction. Among different cell lines MCF-7 was found to be the most susceptible towards ZnO-PEG treatment, followed by HBL-100, MDA-MB and T47D; in the case of MCF-7 more than 25% cell underwent death within 24 h of nanoparticle treatment (Figure 3b). The flow cytometry result further revealed that the cancer cell death is caused by apoptosis and not by necrosis. Apoptosis is programmed cell death, and it is one of the major pathways by which cell dies. Induction of apoptosis normally leads to cell cycle arrest with a reduction in DNA content. A FACS based approached has been utilized here to determine the amount of DNA which has direct correlation with cell cycle propagation. Ethidium bromide (EtBr) is an intercalating agent known to give fluorescence on DNA binding and depending on the DNA content the amount of fluorescence also changes. To identify individual cell population in particular phase, cancer cell staining has been done with EtBr, and when EtBr fluorescence was plotted against total cell population, two well-separated groups were formed localized in G0/G1 and G2/M phases. We have further realized that on nanoparticle treatment there is shift of cancer cell population towards sub G0/G1 indicating possible cell cycle arrest (Figure 3c). Apoptosis is normally associated with changes in phospholipids content of the outer cytoplasmic membrane where as cell death caused by necrosis is associated with non-physiological circumstances that disrupt
cellular homeostasis (e.g., ischemia, hypoxia and poisoning). Necrosis is caused by membrane dissolution (osmotic lysis, shear stress, pore-forming proteins, loss of ATP) as a result of which the cellular material (including degradative enzymes) is released into the surrounding tissue. In apoptotic cells, the membrane phospholipid, phosphatidylserine (PS), is translocated from inner to outer leaflet of plasma membrane, thereby exposing PS to the external cellular environment. This event can easily be tracked by using annexin V-conjugated to fluorescein isothiocyanate (FITC), as annexin V-FITC has high affinity to PS. Due to the large size of annexin V-FITC, the conjugate cannot penetrate inside the plasma membrane, and it will bind only to those cells which have externalized PS and necrotic cells which contain leaky membranes. Counterstaining with PI can distinguish cells on the basis of early and late apoptotic stages. Generally annexin V-FITC positive and PI negative cells were considered to be early apoptotic stage; whereas both annexin V-FITC and PI double positive cells were considered to be in the late apoptotic or necrotic stage. It is a confirmatory test for apoptosis. We found that more than 35% of the total cell population undergoes apoptosis in presence of 25 µg/ml of ZnO-PEG (Figure 3d). This data were further verified using trypan blue assay. The DNA damage induced by ZnO-PEG was analyzed by DAPI staining, the image clearly demonstrates that on nanoparticle treatment occurrence of nuclear DNA aberration and fragmentation was a common event (Figure 3e). The morphological characterization of NP treated cells using light microscopy clearly indicates structural transition. Treated cells have lost their normal cellular morphology; in fact cells become rounded and clumped on NP treatment indicating possible cell dying event (Figure 3f). Similar result was seen when the treated cell was visualized in scanning electron microscopy (SEM), which indicated that the smooth surface characteristics of healthy cells got transformed to rough and irregular one (typical characteristics of dead cell) on nanoparticle treatment (Figure 3g).
Figure 3. (a) Toxicity profile of ZnO-PEG and ZnO on PBMC cells; ZnO-PEG was found to be non-toxic on normal cells. b) Histogram of percent cell death in different breast cancer cell lines upon treatment with ZnO-PEG for 24 and 48 hrs. c) Cell cycle analysis d) annexin-V and 7AAD fluorescence and e) DAPI staining showing mode of ZnO-PEG induced death is apoptosis and not necrosis. Morphological changes observed in cancer cells in presence of NPs captured by f) light microscope and g) SEM showing damage in cancer cells when incubated with NP.

5.7.4. Internalization of Nanoparticle. We have used FITC capped ZnO to monitor the internalization process. Our studies demonstrate that FITC-ZnO is efficiently internalized within the cell, and distributed all over the cell surface, cytoplasm and nucleus (Figure 4ab). The internalization is concentration dependent and the maximum internalization
occurs within the first two hours of nanoparticle treatment. We have also noticed the presence of nanoparticle at the cell surface. Cell membrane normally consists of lipid bilayer, and the fast uptake of any foreign substance generally involves endocytosis. In case of cancer cell we do not observe any appreciable membrane injury on nanoparticle treatment; so the only possibility remains for ZnO-PEG internalization is through receptor mediated endocytosis (Figure 4c).

Figure 4. (a) Differential interference contrast (DIC) and b) confocal images showing PEG-ZnO internalization in MCF-7 cells. c) A cartoon representation showing internalization of ZnO-PEG inside the cancer cell.

In section 5.7.3. we have established that ZnO-PEG induces apoptotic death in different breast cancer cell lines, however, the question remain which apoptotic pathway ZnO-PEG follows to kill the cancer cell. p53 is the global transcription regulator of cancer, and normally nanoparticle prefers to induce apoptosis via p53 pathway. However we have witnessed ZnO-PEG mediated apoptosis even in p53 mutated cell lines (MDA-MB and T47D) thereby raising the possibility of the involvement of p53-independent apoptosis pathway in nanoparticle-induced killing. Apart from p53 the other major pathway that operates in apoptosis process is Fas-FADD and literature evidence
shows that Fas mediated apoptosis is very common in MDA-MB cell line. To gain insight into ZnO-PEG mediated apoptosis, we primarily screen the expression level of different signaling molecules involved in Fas-FADD pathway.

5.7.5. Towards an Understanding the Mode of ZnO-PEG Action

5.7.5.1. Up Regulation of Fas at the Cell Surface of Cancer Cell. We have already found that ZnO is capable the ROS production, and the ROS generation capacity intensifies several folds on polymeric capping (Chapter 3). Direct impact of ROS production and oxidative stress in cancer cell is the up-regulation of the cell surface Fas receptor. Fas (also called Apo1 or CD95) is a death domain-containing member of the TNFR (Tumor Necrosis Factor Receptor) superfamily which has a pivotal role in the physiological regulation of programmed cell death (apoptosis) and has been implicated in the pathogenesis of various malignancies and diseases of the immune system. The Fas Receptor initiates an apoptotic event by binding to FasL or other external ligand. Fas is a Type-I transmembrane protein, whereas FasL is a Type-II trans-membrane protein of TNF family. To check the Fas expression level in our selected cell line we have performed FACS analysis, which shows a significant up-regulation of Fas expression on the surface of cancer cells treated with ZnO-PEG (net mean fluorescence intensity (MFI) is 100 ± 5.5, p < 0.05) compared to untreated control cells (MFI is 10 ± 2, p < 0.04) (Figure 5a). We further cross checked Fas regulation using western blot and qRT-PCR (quantitative real time polymerase chain reaction) and the result of both analyses shows significant up regulation of Fas expression level on the surface of nanoparticle treated cancer cells (Figure 5bc). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and α-actin were used here as house keeping gene (control). The confocal laser scanning microscopy image also shows a hefty increase of Fas receptor on ZnO-PEG treatment (Figure 5d). ROS mediated up-regulation of Fas receptor was further verified using N-acetylcysteine (NAC). NAC is a known ROS quencher and we have found that in presence of NAC Fas expression level reduces several fold (MFI) is 15 ± 3, p < 0.01) (Figure 5c). We have also studied the role of Fas L on ZnO-PEG induced apoptosis. In presence of Fas L we do not find any special effect, the apoptotic death of cells remains the same (~35%). When we treated Fas L inhibitor in the system a decrease in ZnO-PEG mediated apoptosis was observed (Figure 5f).
5.7.5.2. Role of Caspases in ZnO-PEG Mediated Apoptosis

The Fas receptor upon binding to any external ligand trimerizes and induces apoptosis through a cytoplasmic domain called DD (Death Domain) that interacts with several signaling adaptors such as FADD (Fas-Associated Death Domain), Daxx, FAP-1, FLASH (FLICE-associated huge) and RIP (Receptor-Interacting Protein). Among these adaptors, FADD carries a DED (Death Effector Domain) and by homologous interaction it recruits the DED containing procaspase-8 protein which is in the inactive state. This protein complex is also known as DISC (Death-Inducing Signaling Complex) and exists in Type-I cells.\textsuperscript{32,33} Procaspase-8 is proteolytically activated to caspase-8. FADD also helps in the activation of caspase-10. Upon activation, caspase-8 cleaves and activates downstream effector caspases, including caspase-3, 6 and 7. Activated caspase-8 stimulates caspase-3 through two pathways: in the first pathway activated caspase-8 cleaves BID (Bcl2 Interacting Protein), thus generating a truncated form of BID (t-Bid, containing COOH-terminal part).\textsuperscript{34} t-BID translocates to mitochondria where it triggers the release of mitochondrial pro-apoptotic factors, like Cyt-C (Figure 1). The released Cyt-C binds to APAF1 (Apoptotic Protease Activating Factor-1) together with dATP and procaspase-9 and activates caspase-9. Caspase-9 cleaves procaspase-3 and activates caspase-3. In another pathway caspase-8 cleaves procaspase-3 directly and activates it.
Both pathways are regulated at the level of caspase-8 activation by the endogenous inhibitor FLIP (FLICE (FADD Like IL-1β-Converting Enzyme)-Inhibitory Protein), which may also be recruited by FADD. From the above discussion it is quiet clear that Fas expression is tightly regulated with FADD formation. As we have seen the up-regulation of Fas on ZnO-PEG treatment, we studied the status of FADD on NP treatment. As expected, FADD expression also gears up several fold on nanoparticle treatment (Figure 6a). To further verify the role of FADD in ZnO-PEG mediated apoptosis, we have transfected the medium with Dn-FADD. Dn-FADD is a deletion mutant of FADD lacking the DED domain, (Dn-FADD, residues 80–208), is inhibitory to CD95 and tumor necrosis factor receptor 1 (TNFR1)-mediated cell death in many contexts. On Dn-FADD treatment a decrease in apoptosis was observed (Figure 6b), to further confirmed our claim that FADD is actively involved in ZnO-PEG mediated apoptosis pathway. After observing the above signal cascade, we became interested to study the impact of nanoparticle on downstream activators of Fas/FADD, especially the caspases, since they are the key executor of apoptosis. We monitored the change in expression level of procaspase-8 and caspase-8 using western blot and our result shows that there is a large increment in caspase-8 expression on nanoparticle treatment (Figure 6c). We have also checked the expression level of FADD, procaspase-8 and caspase-8 in presence of NAC, and our result shows that the expression level of these proteins decreases significantly on NAC treatment (Figure 6c).

**Figure 6.** (a) Fas-associated FADD activation, detected by western blot analysis from the anti-Fas-purified immune-complex. b) Dn-FADD/control vector-transfected cancer cells were treated with/without PEG-ZnO and percent apoptosis was scored by Annexin-V positivity FITC/7-AAD positivity. c) Expression levels of FADD, pro caspase-8, caspase-8 proteins determined in PEG-ZnO-treated cells incubated with or without NAC by western blot analysis.
Caspase expression is directly related to apoptosis. To validate this claim we plot the percentage change in the expression level of different caspases against cell death and we discover that with increasing caspase expression there is an enhancement in cancer cell death. This phenomenon is obvious as caspases are key regulator of apoptosis. To substantiate the role of caspases in cancer cell apoptosis we deliberately added caspase specific inhibitors in the cell culture medium and found that in all inhibitor treated cancer cells, apoptosis was heavily reduced, again supporting our claim that caspases are playing an important role in ZnO-PEG mediated apoptosis (Figure 7a). The same trend was seen in the western blot analysis (Figure 7b). Bid is a specific substrate of caspase-8 and activation of caspase-8 is associated with cleavage of Bid. We have already seen the activation of caspase-8, thus it is interesting to examine the expression level of Bid and t-Bid upon nanoparticle treatment. Results of Figure 7c depict a gradual accumulation of t-Bid in the cytosolic fraction and simultaneous translocation to mitochondria upon nanoparticle treatment.

Zhai et al have earlier observed that the cleavage of Bid by caspase-8 can lead to the mitochondrial release of Cyt-c. They also speculated that the release of Cyt-c from the mitochondria may occur via the interaction of Bid with mitochondrial surface protein Bax. We also have a similar kind of observation. We confirmed the release of Cyt-c
using western blot analysis (Figure 7c). The release of Cyt-c causes the activation of several caspases including caspase-9. In fact activated caspase-9 and Cyt-c form the apoptosome complex, which in turn stimulates caspase-3 activation. Data represented here clearly suggest the possibility of cross-talk between death receptor pathway and mitochondrial pathway in nanoparticle-triggered apoptosis.

5.7.6. Nanoparticle-Induced Lipid Peroxidation and Change in Membrane Potential ($\Delta \psi_m$) of the Mitochondria

Previously several groups have reported that the internalization of nanoparticle is associated with ROS production and organelle damage. Mitochondria, the cellular powerhouse and one of the key regulators of apoptosis, is very susceptible to ROS generation. It was earlier reported that the internalization of quantum dots (QDs) leads to intracellular ROS accumulation and organelle damage. Here we have identified two intracellular targets of this QD-induced ROS, namely membrane lipids and mitochondria. In response to oxidative stress, cell surface and organellar membrane lipids may undergo peroxidation. Flow cytometry has been utilized here to assess the intracellular ROS level in different cancer cells. We have used fluorescent 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) as ROS substrates for our investigation. Compared to untreated control cells (MFI, 10 ± 2.1%), ZnO-PEG nanoparticle treated cells showed significant enhancement in intracellular ROS production (MFI, 100.0 ± 6.3%). ROS accumulation was also measured at different time points of nanoparticle incubation. A marginal gain in fluorescence intensity was observed in higher time point (MFI at 8 h time point 140 ± 12.5) (Figure 8a). We have also measured ROS in presence of NAC. ROS generation decreases considerably in the presence of NAC (Figure 8b). The generation of ROS was further confirmed using fluorescence microscopy, where DAPI was used as a counter stain (Figure 8c). Direct consequence of ROS production is mitochondrial membrane lipid peroxidation and that causes accumulation of cytotoxic aldehydes. This aldehyde at the mitochondrial level, can impair mitochondrial functions. For determining the MTP (Mitochondrial Transmembrane Potential, $\Delta \psi_m$) loss, cells were loaded with mitochondrial membrane potential-sensitive dye 3,3'-dihexyloxacarbocyanine iodide (DiOC6) and fluorescence of retained DiOC6 was determined flow cytometrically. FACS analysis showed that nanoparticle treated cancer cells furnished a reduced uptake of
DiOC6 (Figure 8d). The effect was reversed on treatment with cyclosporine A (CsA), a known inhibitor of mitochondrial pore formation (Figure 8d). The result reported here suggests that ZnO-PEG mediated apoptosis involves mitochondrial membrane damage and trans-membrane potential loss.

5.8. Discussion

Breast cancer is one of the major causes of morbidity in female. From the therapeutic point of view, it is therefore important to devise strategies to induce apoptosis in breast cancer cells. Literature evidences suggest that various metal and metal oxide nanoparticles have cytotoxicity against different cancer cell lines. These results are provoking and can be utilized in designing the next generation cancer drug. However, most of the nanoparticles are non-specific in nature and indiscriminately kill normal healthy cells which makes them inappropriate for clinical trial. Initial reports on the
potential toxicity of some uncapped nanoparticles prompted the development of differently modified NPs as tools in biological sciences.

Several studies have described surface modifications to improve nanoparticles biocompatibility, for their potential applications in medical sciences. Among various nanomaterial ZnO has the ability to kill cancer cells selectively, although we have seen some toxicity of this material against PBMC cells (Figure 3a). To reduce its toxicity and make it more biocompatible towards normal cells we have purposefully functionalized its surface with PEG. Impressive result was observed on PEG capping over ZnO, reducing NP-mediated toxicity towards PBMC cell significantly (Figure 3a). It was further revealed that the ability to kill cancer cells by ZnO did not alter much on PEG capping. ZnO-PEG is a very promising nano-material which may find suitability in clinical application. However, for any clinical utilization, a clear understanding of the mechanism of nanoparticle action is important. In the present study, we have investigated the molecular mechanisms of ZnO-PEG-mediated apoptosis in both p53-dependent and mutated human breast cancer cell lines.

It is generally accepted that cell death can be the result of either apoptosis or necrosis. Interestingly our result indicates that ZnO-PEG induced cytotoxicity is apoptotic in nature. Apoptosis is a complicated process involving several signaling pathways. The question is which pathway does ZnO-PEG mediated apoptosis follow? In mammals, signaling cascades culminating in apoptotic cell death can be mainly divided into intrinsic or extrinsic pathways. Earlier data suggest that the extrinsic pathway is probably involved in ZnO-PEG mediated apoptosis, although the pathway is not well established. To confirm the mechanism of action we first checked the expression level of major constituents of extrinsic cell signaling pathway, like Fas, FADD, and caspase-8 and our result clearly revealed that on NP treatment there is a heavy increase of cell surface Fas receptor (Figures 5). When we further analyzed our data we interestingly found that not only Fas, but the expression level of several Fas-associated adaptor molecules, such as FADD and caspase-8 was also stimulated significantly on nanoparticle treatment. It is known that the activation of Fas receptor results in the formation of death inducing signaling complex (DISC) through the association of FADD and caspase-8. Normally DISC formation triggers caspases-8 auto-catalysis, which in
turn initiates cascade of signaling events.\textsuperscript{33,42} It has been known that the activation of caspase-8 leads to cleavage of BID and the formation of t-BID. (Bid is a pro-apoptotic Bcl-2 family protein which plays a crucial role in Fas-FADD mediated apoptosis). Here we have also observed a similar phenomenon (Figures 6 and 7). Interaction of BID with mitochondria and the loss of mitochondrial integrity are well known.\textsuperscript{35} Changes in the mitochondrial membrane permeability are considered an early event in apoptosis. The direct consequence of mitochondrial membrane permeabilization is the release of Cyt-c. We have found that ZnO-PEG treated breast cancer cells suffer a massive change in mitochondrial membrane potential (\(\Delta\psi_m\)) (Figure 8d). It is normally believed that the release of Cyt-c into the cytosol leads to caspase-9 activation through the formation of Cyt-c/Apaf-1 complex. Caspase-9 is the upstream caspase in the mitochondria-dependent apoptosis pathway and further activates caspase-3, which ultimately results cell death (Figure 1).

5.9. Conclusion

Nanoparticle biocompatibility can be easily altered by surface modifications, such as conjugation and capping with biomolecules and polymers. Here we have introduced the surface functionlization of NP through PEG ligation to make it more biocompatible. In summary it was found that ZnO-PEG nanoparticle is an efficient killer of cancer cells. It induces apoptosis in cancer cell through Fas-FADD pathway. Fas-FADD activation ultimately results in cleavage of BID and alteration of mitochondrial transmembrane potential that leads to Cyt-c release and activation of several caspases (Figure 9). Although this is the major pathway involved in cancer cell apoptosis, we could not rule out the possibility of ROS mediated intrinsic apoptosis pathway. Our finding also suggests that PEG-functionalized ZnO nanoparticle is least toxic towards normal PBMC cell. Overall this investigation has shed light on the mechanism of antitumor action of PEG-functionalized ZnO nanoparticle, which may find applicability in the future development of anticancer drug.
Figure 9. Cartoon representation showing the mechanism of ZnO-PEG mediated apoptosis in cancer cell.
5.10. References


