CHAPTER 3

Bactericidal Effect of Polyethyleneimine Capped ZnO Nanoparticle on Multiple Antibiotic Resistant Bacteria bearing Genes of High Pathogenicity Island

Abstract: Zinc oxide nanoparticles (ZnO-NP) were synthesized by alcoholic route using zinc acetate as precursor material and lithium hydroxide as hydrolyzing agent. Further ZnO-PEI (ZnO derivatives) was made in aqueous medium using capping agent polyethyleneimine (PEI). X-Ray Diffraction (XRD) measurement confirmed the formation of ZnO NP with hexagonal wurtzite structure. The average size of PEI functionalized ZnO (3-7 nm) was determined by using Scherer’s equation and confirmed by transmission electron microscope (TEM). ZnO-PEI is water soluble and forms colloidal suspension in water. These functionalized nanoparticles along with uncapped ZnO showed promising antibacterial activity against a range of multidrug resistant bacterial species. ZnO effectively killed these microorganisms by generating reactive oxygen species (ROS). A synergistic effect was further seen when nanoparticle was co-treated with tetracycline.

3.1. Mechanism of Action of Antibiotics

The discovery of penicillin in 1928 by Sir Alexander Fleming has shown the direction towards the synthesis of new generation of antimicrobial.

The social impact of antibiotic discovery is enormous; and we now take for granted that any infectious disease is curable by antibiotic therapy. Presently antibiotics are manufactured at an estimated scale of about 100,000 tons annually worldwide.\textsuperscript{1} Antibiotics work in variety of ways summarized in Figure 1.

Some antimicrobial agents inhibit bacterial cell wall synthesis. These agents include $\beta$-lactam compounds such as penicillins (e.g. penicillin G, ampicillin and methicillin), cephalosporins and carbapenems, as well as monolactams and $\beta$-lactamase inhibitors. $\beta$-lactams inhibit the final stage of murein bio-synthesis. This, by some unexplored mechanism, triggers murein hydrolases to lyse the cell wall. A related group of antibiotics that prevent a different step in cell wall formation are the glycopeptides, vancomycin and teicoplanin. Other agents have an antibacterial effect by inhibiting protein synthesis. Representatives of this group include the aminoglycosides, tetracyclines, macrolides and chloramphenicol which interfere with ribosome function. In addition, there are certain antibiotics which inhibits DNA synthesis, including quinolones, fluoroquinolones and sulfonamides.\textsuperscript{2}

3.2. Antibiotic Resistance

The bacteria have the potential to develop resistance against antibiotics. However, the continuous emergence bacterial resistant strains are threatening human health and its control is the major challenge for scientists and researchers. World is facing a global challenge of increasing antibiotic resistance,\textsuperscript{3} owing to wide and often indiscriminate use of antibiotics and pesticides and related compounds in agriculture. Once the bacteria come into contact with antibiotic, but not killed by it, bacteria can develop resistance by changing their cell structure or metabolism to destroy the antibiotic in future. Hence, bacterial exposure to antimicrobials is the opportunities for bacteria to acquire resistance. There are a few methods for acquiring resistance, which includes genetic mutation, modification of genetic material or gaining of new genetic material. Once resistance is acquired by bacteria, it can share, exchange and transfer vertically (to its progeny) or horizontally (to neighbouring bacteria) by transduction (bacteriophage-mediated transfer of DNA between more than one bacteria), transformation\textsuperscript{4} (DNA are taken up by bacteria from the external environment) or by conjugation (direct cell-to-cell contact to transfer DNA). If a bacterium carries several resistance genes, it is called multidrug resistant.
(MDR) or, informally, a superbug or a super bacterium. The terminology “MDR” is most frequently used for Gram-positive and Gram-negative bacteria when they are ‘resistant to three or more antimicrobial classes’. MDR-bacterial cell arises during treatment of fully sensitive bacteria when the course of antibiotics is interrupted and the levels of drug in the body are insufficient to kill 100% of bacteria. This phenomenon can happen for a number of reasons: patients may feel better and halt their antibiotic course, drug supplies may run out or become scarce, or patients may forget to take their medication from time to time. MDR infection spreads rapidly from one person to another and it is very difficult to cure and as a result infection occurs very frequently. MDR outbreak among people with weakened immune systems (e.g., patients with HIV) is very common although outbreaks among non immuno-compromised healthy people do occur. Multidrug resistance in bacteria may be generated by one of two mechanisms. First, these bacteria may accumulate multiple genes, each coding for resistance to a single drug, within a single cell. This accumulation occurs typically on resistance (R) plasmids. Second, multidrug resistance may also occur by the increased expression of genes that code for multidrug efflux pumps, eXcluding a wide range of drugs.

3.3. Bacterial Resistance and Nanoparticle

Nanoparticles have been found to be effective against MDR bacterial strains for a long time. Silver is the most utilized nanoparticle against MDR strains. Several groups of researchers have firmly established that silver nanoparticles are the powerful weapons against the MDR bacteria, such as Pseudomonas aeruginosa, ampicillin-resistant Escherichia coli, erythromycin-resistant Streptococcus pyogenes, methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistant Staphylococcus aureus (VRSA). In addition to these, Namasivayam et al. have recently reported the efficacy of silver nanoparticles against multidrug resistant Klebsiella pneumoniae. Although silver NP is effective against most of the common MDR bacteria, its application is limited since it has severe toxic side effect. Apart from silver, Copper oxide (CuO), Titanium dioxide (TiO₂) and Iron oxide (Fe₂O₃) nanoparticles have also found promising application against MDR bacterial strains and Saccharomyces cerevisiae. Although Ag is the most utilized antimicrobial nanoparticle, the use of ZnO as an alternative agent is escalating because of its biocompatibility and robustness. It has been used for long time in food
processing industry as an antimicrobial agent. Recent studies have shown that these nanoparticles have selective toxicity towards bacteria, but exhibit minimal effects on human cells.\textsuperscript{11} It has been reported that ZnO nanoparticles possesses antibacterial activities against both Gram-positive and Gram-negative bacteria, including major food borne pathogen, such as \textit{Escherichia coli} O157:H7, \textit{Salmonella}, \textit{Listeria monocytogenes}, and \textit{Staphylococcus aureus}.\textsuperscript{10,12} Although the antimicrobial potency of zinc oxide has been explored against several Gram-positive and Gram-negative bacteria, the application of this material against MDR bacterial strain has just begun.\textsuperscript{10} The first evidence of ZnO action against multidrug resistance \textit{Streptococcus aureus} was come from Jones et al,\textsuperscript{13} who found that the killing ability of zinc oxide against multidrug resistance cells increased in presence of some common antibiotics such as ciprofloxacin. Although the combination therapy is found useful against multidrug resistance bacteria, unfortunately the exact mechanism of nanoparticle action still remains ambiguous.\textsuperscript{14}

3.4. The Antimicrobial Mechanism of Action of Zinc oxide NP

Although earlier studies have assessed the antibacterial properties of ZnO NPs, the precise mechanism is still elusive. However, a number of studies have indicated that the primary cause of the antibacterial action might be from the disruption of cell membrane.\textsuperscript{15} Another possibility could be the induction of extracellular and intracellular reactive oxygen species, including hydrogen peroxide (H$_2$O$_2$),\textsuperscript{16} a strong oxidizing agent harmful to bacterial cells. It has also been reported that ZnO can be activated by UV and visible light to generate increasing amount of reactive oxygen species such as OH$^\cdot$, H$_2$O$_2$, and O$_2$.\textsuperscript{17} Among these variety of radicals, the negatively charged hydroxyl radicals and superoxide cannot penetrate inside the cell membrane and are likely to remain on the cell surface, whereas H$_2$O$_2$ can penetrate inside the bacterial cells.\textsuperscript{18} The third possibility could be the release of toxic Zn$^{+2}$ from the dissolution of NP.\textsuperscript{9,19}

3.5. Safety Issue

Nanoparticle toxicity is an important issue which should be address before any bio-application. Although silver and zinc oxide nanoparticles share many similarities like broad range of antimicrobial activity,\textsuperscript{20} a variety of synthesis method etc; but their toxicity against normal human cell line is remarkably different. Silver is found to be more
toxic towards human cell compare to zinc oxide.\textsuperscript{10} Zinc oxide has very little toxicity in bulk size that is why it is termed as GRAS (Grossly Recognized as Safe) by FDA (Federal Drug Administration). Zinc oxide was widely used as an active ingredient for dermatological application (cream, lotion and ointments) and food product on accounts of its antibacterial application, and impressively there was no report of toxicity.\textsuperscript{21} The nontoxic nature of zinc particle has tempted us to use it as an alternative of antibiotic.

3.6. Surface Functionalization and its Effect on Antimicrobial Potential

Polymeric agents have long been used to improve the dispersion of NPs in biological fluid. It has been shown that polyvinyl alcohol (PVA) and chitosin capping over ZnO improved its antimicrobia. efficacy due to better dispersion of the nanoparticle in biological medium.\textsuperscript{15,22} Polyethyleneimine (PEI) is an aliphatic, cationic branched polymer containing primary, secondary and tertiary amines in its structure. PEI is mostly known for its transfection ability. Among the polymer-based formulation for gene transfection, PEI is one of the most efficient polymers for non-viral gene delivery applications and its ability to transfect a wide range of cells is well established.\textsuperscript{23} Apart from these applications PEI can also be used as an efficient membrane permeabilizing agent for Gram-negative bacteria. Although the membrane intervening ability of PEI has been known for quite some time, the inherent antimicrobial potency of this material has recently been established.\textsuperscript{24} It was found that the quaternary polyethyleneimine NP has long-lasting antibacterial action against the cariogenic \textit{Streptococci mutans}.\textsuperscript{25} PEI capping also improves silver NP colloidal stability and the antimicrobial activity.\textsuperscript{26} In spite of these qualities, the toxicity of PEI always remains an issue. However, a recent study has demonstrated that the careful selection of the size of PEI polymer greatly reduces the toxicity emanating from its cationic nature. It has been shown that 10 kDa PEI was particularly efficient in the safe delivery of siRNA and DNA constructs with minimal or no cytotoxicity, in addition to the regular delivery of hydrophobic anticancer drug, paclitaxel, to pancreatic cancer cells.\textsuperscript{27} According to another study involving FEI cells, neither PEI-polymer nor nano ZnO crystals could elicit any significant mutagenic activity or oxidative DNA damage in the exposed cells, suggesting their safe use in clinical trials.\textsuperscript{28} To enhance the antimicrobial activity and stability of nanoparticle in biological fluid we deliberately used PEI capping over ZnO and targeted it against MDR
bacteria. The rational behind designing such nanoconjugate is to enhance the internalization rate of NP within the bacterial cells as it has been already established that PEI can specifically target lipopolysaccharide present in the Gram-negative bacteria cell wall.29

3.7. Motivation of Present Work

The re-emergence of infectious diseases and the continuous development of multidrug resistant strains among a variety of disease-causing bacteria is a serious threat to public health worldwide.3 Despite conventional antimicrobial therapy, morbidity and mortality associated with these infections remains very high. The action of synthetic antibiotic is often restricted to certain family of bacteria, which helps microorganisms to develop resistance against all antibiotics. New strategies are therefore needed to identify and develop the next generation of drugs or agents to control bacterial infections. One of the growing approaches is the use of metal and metal oxide nanoparticle as an alternative of antibiotic.6 In this investigation, ZnO-NP was synthesized, characterized and targeted against several enteropathogenic MAR (Multiple Antibiotic Resistance) bacteria containing HPI (High Pathogenicity Island) genes. Membrane and DNA damaging potential of ZnO NP were also assessed using various imaging techniques.

3.8. Materials and Methods

3.8.1. 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 [Zn(CH3COO)2·2H2O], lithium hydroxide monohydrate (LiOH·H2O), ethanol and n-hexane.30 All reagents used were of analytical grade. 10 mM [Zn(CH3COO)2·2H2O] 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 QDs were precipitated using n-hexane and centrifuged. The ZnO QD powder was obtained by washing the precipitate with ethanol and dried at 60 °C.30 The synthesized ZnO surfaces were modified with capping agents for different biological applications.

3.8.2. Polyethylenimine (PEI) Capping. To the freshly prepared ZnO QD solution, 0.01 M of trisodiumcitrate dihydrate was added with continuous stirring. The resulting
solution was centrifuged and washed with ethanol/water. The collected precipitate was dispersed in aqueous solution of 60 ml of PEI and stirred for 6 h in order to facilitate the efficient capping of PEI. The PEI-functionalized ZnO NPs (ZnO-PEI in short) were then collected by centrifugation, washed with water and dried at 50 °C to obtain the powder.31

3.8.3. Characterization of NP by 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, sonicated sample solutions on a carbon-coated copper grid and dried completely in dust free atmosphere. The bright field electron micrographs of the samples had been recorded on JEM-2010 (device: Orius SC1000) at the accelerating voltage of 200 kV.

3.8.4. FTIR Spectroscopy. FTIR technique was used to determine the binding of PEI 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.

3.8.5. Atomic Force Microscopy. To determine the morphology of PEI-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 Si₃N₄ tip with resonance frequency of 100 kHz and spring constant being 0.6 N m⁻¹ to obtain surface topography of deposited ZnO-PEI NPs. The film was air dried in dust free environment for measurement.

3.8.6. Isolation of Multiple Antibiotic Resistant Bacteria

3.8.6.1. Sampling. Composite water samples were collected following standard methodology (American Public Health Administration, APHA, 1989) from a single sampling station on Mahananda River, West Bengal. The samples were collected in heat-sterilized, screw-capped bottles, transported on ice, and processed within 6 h of collection.
3.8.6.2. Isolation of Multiple-Antibiotic Resistant Bacteria. Serially diluted water samples were spread on McConkey, Eosin Methylene Blue (EMB), Xylose Lysine Deoxycholate (XLD), and Hektoen Enteric agar (HiMedia, India) plates and incubated at 37 °C. Replica plating method was done for determining the antibiogram of the isolates. Multiple-antibiotic resistant (MAR) isolates were screened as per EUCAST guidelines (http://www.eucast.org/clinical-breakpoints).

3.8.6.3. Antibiotic Susceptibility Test The susceptibility to antibiotics was tested as described previously32 (http://www.escmid.org/fileadmin/src/media/PDFs), as described by the European Society of Clinical Microbiology and Infectious Disease (http://www.escmid.org/). Escherichia coli ATCC 25922 was used as control. Criteria for susceptibility or resistance followed the EUCAST guidelines (http://www.eucast.org/clinical-breakpoints). Susceptibility to the antibiotics absent in EUCAST breakpoints table (v 1.1 2010-04-27), were interpreted according to previously described criteria. Isolates were maintained by bi-weekly transfer to LB agar slants.

3.8.6.4. Selection of MAR Isolates Bearing Genes of High Pathogenicity Island (HPI) MAR isolates were then subjected to PCR assay for detection of irp2 and fyuA genes of the core region of High Pathogenicity Island (HPI). Primers for fyuA gene were 5’-ggeggtgcgttcagc-3’ and 5’-cggtgccgaggttta-3’; and (ii) for irp2 gene, FP (forward primer) 5’-aaggattcgctgttaccggac-3’, RP (reverse primer) 5’-tcgtcgggcagcgtttcttctt-3’. The PCR reaction for fyuA and irp2 contained 2.5 μl 10X PCR buffer; 200 μmol/l each dNTP; 1U Taq Pol, 25 pmol of each of both forward and reverse primers of the genes fyuA and 7.5 pmol of irp2 was used. To that 7.5 μl of template DNA, obtained from boiling lysis of overnight cultures of the isolates, was added. The final volume was made to 25 μl using sterilized distilled water. PCR amplification was done using Bio-Rad DNA Engine, Peltier Thermal Cycler. The following cycling parameters were used for fyuA amplification: 5 min initial denaturation at 95 °C and 30 cycles of denaturation at 95 °C for 1 min; annealing at 58 °C for 1 min and extension at 72 °C for 2 min followed by a final elongation step at 72 °C for 7 min. The following conditions were used for irp2 amplification: denaturing for 7 min at 94 °C, and 35 cycles of 1 min at 94 °C, 1 min at 63 °C and 1 min at 72 °C and a final elongation step for 7 min.
at 72 °C. Amplicons were observed after electrophoretic separation in 1.5% agarose gel. Three strains, YSI6A, HPM13 and MREC33 were selected for this study.

3.8.7. Characterization of the Test Strains

3.8.7.1. Biochemical Characterization. Indole production, lysine utilization, ornithine utilization, urease production, deamination of phenylalanine, reduction of nitrate, H₂S production, citrate utilization, MR test, VP test, malonate utilization and carbon source utilization/fermentation tests were carried out using HiBio-ID/HiCarbo system according to manufacturer’s instruction (HiMedia, Mumbai, India). E coli K12 was used as control.

3.8.8. Characterization of Virulence Properties

3.8.8.1. Blood Hemolysis and Serum Resistance Assay. Human blood was obtained from healthy volunteers. The blood was centrifuged at 1600 rpm for 5 min. The plates containing 0.6% human blood were streaked with HPI positive isolates and incubated at 37 °C for 24 h. The rapid assay for determining the serum resistance phenotype of the bacteria was then performed.

3.8.9. Agglutination Assay

3.8.9.1. Presumptive Agglutination Assay. A presumptive agglutination assay was performed on glass slides following standard method. Drops of serum (1:10 dilution) were placed on glass slides and mid-log bacterial cells were added onto the diluted serum. The slides were then visualized under the phase contrast microscope.

3.8.10. Confirmatory Agglutination Assay. 1 ml of the diluted serum was added aseptically to a tube that contained 1 ml of normal saline. To this 200 μl aliquots of cell suspension from mid-log phase were added. The tubes were kept at 37 °C for 2 h and then allowed to stand at room temperature for 24 h. The tubes that showed visible clumping at the bottom were taken to be positive and the tubes that showed uniform distributed growth were identified as negative.

3.8.11. Swim Motility. Minimal swim motility agar plates containing tryptone 10 (g l⁻¹), NaCl, 5(g l⁻¹), and 0.3% (w/v) Bacto agar were made and the HPI positive isolates were inoculated as a point on the plates and incubated at 30 °C overnight. The motile organisms grew on a larger surface area (thus showing motility) as compared to the non-motile isolates.
3.8.12. Multiplex PCR Assay for Determining the Virulence Factors. Mid-log phase culture was suspended in 200 μl of sterile distilled water for boiling lysis; 5 μl of this was used as the template in 25 μl reaction mixture for amplification. 1 unit Taq polymerase (Genel) was used for the amplification, and the initial denaturation time was 5 min. The isolates were then subjected to multiplex PCR following standard reaction conditions. The primer pairs for genes coding for heat stable toxin ST (stla) and heat labile toxin LT (elt) of ETEC (enterotoxigenic Escherichia coli) and (uidA) of β-glucuronidase were used in Assay no. 1 (n1). Assay no. 2 (n2) was specific for detection of eae (structural gene for intimin found in EPEC (enteropathogenic Escherichia coli) and hfpA (structural gene for bundle-forming pilus) of EPEC, and assay no. 3 (n3) screened for the presence of stx1 and stx2 of shiga toxin producing EHEC (enterohemorrhagic Escherichia coli), and ial (invasion-associated locus of the invasion plasmid) found in EIEC (enteroinvasive Escherichia coli)/ Shigella. Primers of variant gene stlb coding for heat stable toxin of ETEC were included in separate assay no. 4 (n4) together with uidA gene. Two plasmid genes of EAEC (enteroaggregative Escherichia coli) namely aat and aggR were detected simultaneously in assay no. 5 (n5). Amplicons were observed after electrophoretic separation in 2% agarose gel.

3.8.13. Preparation of Nanoparticle Dispersion. Test ZnO nanoparticles (NPs) (ZnO-Ac QD and ZnO-PEI) were suspended in sterile de-ionized water and briefly sonicated by means of ultrasonic-homogenizer (LABSONIC®, Germany), to prevent any aggregation of nanoparticle.

3.8.14. Growth Kinetic Studies of the Test Strains in Presence of Nanoparticles. Varied concentrations of nanoparticles (50, 100 and 150 μg/ml for ZnO and 10, 15, 25 and 35 μg/ml for ZnO-PEI) were tested against the three different MAR bacteria (MREC33, YSI6A and HPM13) in liquid Luria-Bertani (LB) medium in order to examine time dependent growth inhibition in presence of NPs; the minimum inhibitory concentration (MIC) and LD50 concentration (where 50% bacterial growth was inhibited) were determined. Bacterial cultures were inoculated from freshly prepared slants into 10 mL of medium. Aliquots of 0.2 mL from the stock bacterial suspension (10^7 CFU/mL) were added to 100 ml Erlenmeyer flasks containing 10 ml LB broth with varied concentration of NPs and incubated at 37 °C in a rotary shaker (150 rpm). Growth was
measured at different time intervals in terms of absorbance at 600 nm. All the experiments were carried out in triplicate and mean value was reported.

3.8.15. Live/Dead Viability Assay. E. coli MREC33 culture grown to logarithmic phase in LB medium was treated with NP. Following exposure, the impact on bacterial membrane integrity was assessed using a Live/Dead BacLight bacterial viability kit (Invitrogen) as instructed by the manufacturer. To quantify the relative numbers of live and dead cells, the relative fluorescence intensities were measured using a fluorescence plate reader (excitation at 435 nm, emission at 525 and 625 nm).

3.8.16. Scanning Electron Microscopic (SEM) Studies. SEM has been used as a tool to characterize surface features of the bacteria on NP treatment. Overnight grown culture (10^8 CFU/ml) was washed and resuspended in PBS, incubated with NP for 6 h at 37 °C. After incubation cells were fixed with 2% glutaraldehyde and dehydrated with series of ethanol treatments and examined by SEM (FEI Quanta-200 MK2) with an accelerating voltage of 20 kV. Multiple fields of visions were viewed at different magnifications.

3.8.17. Bacteriolytic Effect of ZnO-PEI Nanoparticle. If ZnO-PEI NPs induce damage to MREC33, comparative protein profiling studies of MREC33 culture with or without the NP provide the extent of damage. 1 ml of overnight grown E. coli MREC33 culture was washed and resuspended in PBS and incubated with or without ZnO-PEI NPs for 12 h at 37 °C. After incubation, cells were centrifuged (10000 rpm, 4 °C for 5 min). The supernatants were then analyzed spectrophotometrically (O.D. at 280 nm). The bacterial pellets obtained were again re-suspended in PBS and subjected to sonication to break the bacteria. The bacterial lysate of respective bacterial cells pellets was collected by centrifugation to measure residual protein inside the cell spectrophotometrically (O.D. at 280 nm). The collected protein samples were subjected to 12% SDS-PAGE. The gel was stained by Coomassie brilliant blue R-250 and observed for the protein profile and comparison was made between the control and the treated E. coli MREC33 proteins.

3.8.18. Determination of Extracellular ROS by XTT. XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assay is used to measure the extracellular (reactive oxygen species) ROS generated by ZnO. In presence of superoxide radical the dye XTT is reduced to form a colour adduct (XTT-formazan). A fixed concentration of ZnO and its different capped analogues (35 μg/ml) were incubated in
presence 10 μL XTT. After a fixed time of incubation the suspension was centrifuged and the supernatant was taken for spectroscopic measurement.

3.8.19. Determination of Intracellular ROS in Bacterial cells. Intracellular ROS was measured using dichlorofluorescein diacetate (DCF-DA). Bacterial cells were grown up to an OD₆₀₀ value of 0.5 in LB medium and then incubated with 30 μg/ml of DCF-DA for 30 min at 37 °C under shaking conditions. DCF-DA loaded cells were then treated with 100 μg/ml of ZnO-PEI for 30 min. Cells were then pelleted and resuspended in PBS, and fluorescence values were either measured at an excitation wavelength of 485 nm and emission wavelength of 528 nm using a spectrofluorometer or visualized under a fluorescent microscope.

3.8.20. Effect of Histidine on Antibacterial Effect of ZnO-PEI Nanoparticle. ZnO-NPs are capable of generating ROS in water suspensions. To check whether generation of ROS is one of the mechanisms of antibacterial activity of ZnO-PEI, growth was determined in terms of OD (600 nm) at different time intervals in presence and absence of 5 mM histidine (used here for ROS scavenging) supplemented with or without 15 μg/ml of ZnO-PEI.

3.8.21. DNA Fragmentation Analysis. Genomic DNA of E. coli MREC33 was incubated for 8 h at 37 °C with 25 μg/ml of ZnO-PEI NP. The DNA was then evaluated on 0.8% agarose gel using ethidium bromide as staining agent, and the DNA pattern generated was documented by a gel documentation system.

3.8.22. Synergistic or Additive Effect of ZnO-PEI Nanoparticle. To establish the synergistic or additive effect of ZnO-PEI with tetracycline, E. coli MREC33 cells were grown in flasks each containing 10 ml liquid LB supplemented singly with 20 μg/ml tetracycline, 15 μg/ml ZnO-PEI (LD₉₀ concentration), and the combination of 15 μg/ml ZnO-PEI + 20 μg/ml tetracycline, respectively. Flask containing only inoculums and LB broth, devoid of tetracycline or ZnO-QDs was taken as negative control and flask containing liquid LB medium supplemented with tetracycline and ZnO-PEI was taken as positive control. Growth was determined by measuring optical density (OD) at 600 nm. Disk diffusion method was also used to assay synergistic effect of antibiotics with ZnO-PEI nanoparticle for bactericidal activity against E. coli MREC33 strain on LB agar plates. For this a single colony of test strain was grown in LB broth at 37 °C for 12 h. 1%
inoculums was transferred into a fresh 10 ml liquid LB medium. Mid-log cells were applied to the plates along with Octodisc (Himedia, India) impregnated with ZnO-PEI. Octodiscs impregnated with only 10 μl of sterile double distilled water instead of ZnO-PEI were taken as control. Antibiotics tested for synergistic effect were Ampicillin (A), 10 μg; Cephalothin (Ch), 5 μg; Colistin sulphate, (Cl) 25 μg; Gentamicin (G), 10 μg; Streptomycin (S), 10 μg; Sulphatriad (Sl), 200 μg; Tetracycline (T), 25 μg; and Co-Trimoxazole (Co), 25 μg per disc respectively. To determine the synergistic effects, each standard disk was added with 10 μl of ZnO-PEI (final concentration of 15 μg ml⁻¹).

3.8.23. Interaction of ZnO-PEI Nanoparticle with BSA. The flasks containing liquid LB supplemented with or without 35 g/l concentration of BSA was inoculated (10⁸ CFU/ml) with freshly prepared bacterial suspension of *E. coli* MREC33. Flasks were allowed to grow for 4 h at 37 °C. After 4 h of incubation, aliquots of cultures were withdrawn, serially diluted in saline water, plated (LB agar) and incubated overnight at 37 °C for enumerating the initial viable cell number (before addition of ZnO-PEI NP). To the above 4 h cultures (BSA supplemented and without BSA) ZnO-PEI NP at complete inhibitory concentration (growth inhibition > 95%) of 35 μg/ml was added and allowed to grow for further 12 h at 37 °C. Aliquots of cultures were withdrawn, serially diluted and plated on LB agar for enumerating the final viable number of cells. Taking the initial count of viable cells as 100%, the survivability or % killed was then calculated from the final count.⁴⁶

3.9. Results

3.9.1. Toxicity of Nanoparticle.

Peripheral blood mononuclear cell (PBMC) red blood corpuscle (RBC) and MCF-7 cells were used here to check the toxicity of ZnO-PEI. Different toxicological assays have been used to determine IC₅₀ value of ZnO-PEI. For example, toxicity of ZnO-PEI against PBMC and MCF-7 was calculated from the rate of apoptosis using some standard fluorescence activated cell sorter (FACS) based assay (details in Chapter 4). Hemolysis rate was measured in case of RBC to determine the toxicity of ZnO-PEI. In all the cases a dose-dependent cytotoxicity was seen and the IC₅₀ values also vary depending upon the type of cell line. The variation in IC₅₀ value is quiet expected as ZnO produces different amount of ROS in different cell lines. A ZnO-PEI concentration less than 25μg/ml are
found to be safe against both the cell lines used here (Figure 2). So a concentration of ZnO-PEI below 25 μg/ml is considered to be toxicologically safe.

![Figure 2](image)

**Figure 2.** Toxicity of ZnO-PEI against different human cell lines (a) PBMC (peripheral blood mononuclear cell), (b) RBC (red blood corpuscle). In (b) tube 2 is the positive control (100% hemolysis); tube 4 is normal control, where NP is absent and tubes 1, 3 represent RBC at varying concentration of NP.

### 3.9.2. Synthesis and Characterization of ZnO-PEI

The ZnO NPs were prepared according to the modified sol-gel route, subsequently modified with trisodium citrate, followed by (PEI) capping (Figure 3a). The detailed synthesis is described elsewhere. NPs were characterized using high resolution transmission electron microscopy (HRTEM) and Fourier transform infrared spectroscopy (FTIR). Figure 3b shows the TEM micrograph of water dispersed ZnO-PEI NPs at 20 nm scale and the average range of core particle size is ~3-7 nm. High resolution AFM images of ZnO-PEI NPs displaying the surface morphology are shown in Figure 3c. Usually the surface morphology studies do not provide the individual particle size; rather they show the average grain size at deposited film surface. It can be seen that the spin casted ZnO-PEI exhibits size dispersity containing both large and smaller grains. The smaller grain size covers the range of 10-15 nm, while the larger grain size is in 45-65 nm range, though the average height of grains is nearly 20 nm. The surface morphology measurements suggest that the spin casting increases the grain size of ZnO-PEI at silicon surface as compared to the dispersed small particles in solution. The presence of PEI on the surface of ZnO was further confirmed by FTIR (Figure 3d), which shows twin peaks at 1587 and 1388 cm\(^{-1}\) and a broad peak at 3200-3400 cm\(^{-1}\) region coming from PEI. The sharp peak at 1587 cm\(^{-1}\) is assigned to N-H bending mode of the amine group overlapped with the C-H bending mode of the methylene (-CH\(_2\)) group. The peak at 1388 cm\(^{-1}\) is due
to the C-H bending vibration of the methyl (-CH₃) group. The symmetric and asymmetric stretching modes of N-H appear at higher frequency located around 3389 and 3250 cm⁻¹. The FTIR spectrum also has a typical metal oxide (Zn-O) peak at 465 cm⁻¹, indicating the formation of ZnO.

3.9.3. Characterization of the Antibiotic Resistance of the Test Strains

As per EUCAST (www.eucast.org) criteria for defining antibiotic resistance (R) /susceptibility (S), the test strains were resistant to multiple antibiotics; Strain YS16A was resistant to ampicillin (R > 50 mg/L) and cefepime (S≤R> 1/4 mg/L) tetracycline (R>20 mg/L) and azithromycin (R>10 mg/L); HPM13 was resistant to ampicillin(R > 50 mg/L), azithromycin(≤ 16 mg/L), cefepime (S≤R> 1/4 mg/L), ciprofloxacin (S≤R> 0.5/1), levofloxacin (S≤R< 1/2), sulfamethoxazole (R≥15 mg/L), and tetracycline (R≥20
mg/L). MREC33 was resistant to ampicillin (R > 50 mg/L), azithromycin (≤ 16 mg/L), cefepime (S/R > 1/4 mg/L), cefotaxime (S/R > 1/4), ciprofloxacin (S/R > 0.5/1 mg/L), kanamycin (R≥10 mg/L), levofloxacin (S/R > 1/2 mg/L), netilmicin (S/R > 2/4 mg/L), sulfamethoxazole (R≥15 mg/L), streptomycin (R≥2.5 mg/L) and tetracycline (R≥20 mg/L).

3.9.3.1. PCR Screening of MAR Strains to Locate HPI Specific Genes, irp2 and fyuA

Amplicons were produced with primer pair(s) specific for irp2 and fyuA gene(s) which corresponded to the reported sizes (~208 and ~ 209 bp for irp2 and fyuA specific amplicon, respectively) in all the three test strains (Figure 4).

Figure 4. Agarose gel electrophoresis (1.5%) of PCR-amplified irp2 and fyuA genes of the HPI positive isolates.

3.9.4. Characterization of the Test Strains

On EMB agar plates E.coli strain MREC33 produced dark blue black colonies with metallic green sheen, while Klebsiella pneumoniae strain YSI6A produced brown, dark-centered and mucoid colonies. On Mac Conkey agar plates, HPM13 produced colorless colonies. HPM13 also produced red colonies on XLD agar plates, and displays green and moist colonies on Hektoen enteric (HE) agar surface. HPM13 and MREC33 produced indole and were negative to Voges-Proskauer (VP) and a citrate utilization test, while
YSI6A was unable to produce indole and was positive to VP and utilized citrate. A comparative table (Table 1) has been prepared on the basis of the identification index of various *Enterobacteriaceae* species supplied with Hi25TM *Enterobacteriaceae* Identification kit (HiMedia, India). The isolates HPM13, YSI6A and MREC33 had > 95% similarity with *Shigella sonnei*, *Klebsiella pneumoniae* and *Escherichia coli*, respectively. MREC33 was found positive to haemolysis, agglutination, swim motility, and serum resistance tests.

**Table 1.** Results of carbohydrate utilization and biochemical tests shown by the test strains compared with the identification index of *Enterobacteriaceae* species that resembled most with the respective test strain

<table>
<thead>
<tr>
<th>No.</th>
<th>Test</th>
<th>HPM13</th>
<th><em>Shigella sonnei</em></th>
<th>YSI6A</th>
<th><em>Klebsiella pneumoniae</em></th>
<th>MREC33</th>
<th><em>E. coli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Esculin</td>
<td>_</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>V</td>
</tr>
<tr>
<td>2.</td>
<td>Arabinose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3.</td>
<td>Xylose</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4.</td>
<td>Adonitol</td>
<td>_</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5.</td>
<td>Rhamnose</td>
<td>_</td>
<td>V</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>V</td>
</tr>
<tr>
<td>6.</td>
<td>Cellobiose</td>
<td>_</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7.</td>
<td>Melibiose</td>
<td>_</td>
<td>V</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>V</td>
</tr>
<tr>
<td>8.</td>
<td>Saccharose</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>V</td>
</tr>
<tr>
<td>9.</td>
<td>Raffinose</td>
<td>_</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>V</td>
</tr>
<tr>
<td>10.</td>
<td>Trehalose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11.</td>
<td>Glucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12.</td>
<td>Lactose</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>13.</td>
<td>ONPG</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14.</td>
<td>Lysin Decarboxylase</td>
<td>_</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>15.</td>
<td>Ornithin Decarboxylase</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>V</td>
</tr>
</tbody>
</table>
V = 11-89% positive

The isolates, HPM13 and YSI6A were also positive to all tests except hemolysis test in case of YSI6A and agglutination test in case of HPM13. Results of the multiplex PCR assays are summarized in Table 2. The assay revealed that YSI6A contains the least number of virulence genes. n1 and n4 assays showed that MREC33 and HPM13 displayed the presence of *uidA*. n2 assay showed the presence of *eae* only in YSI16A and

<table>
<thead>
<tr>
<th>Multiplex Assay</th>
<th>n1</th>
<th>n2</th>
<th>n3</th>
<th>n4</th>
<th>n5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolates</td>
<td>elt</td>
<td>stla</td>
<td>uidA</td>
<td>eae</td>
<td>bfpA</td>
</tr>
<tr>
<td>HPM13</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YSI6A</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MREC33</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The isolates, HPM13 and YSI6A were also positive to all tests except hemolysis test in case of YSI6A and agglutination test in case of HPM13. Results of the multiplex PCR assays are summarized in Table 2. The assay revealed that YSI6A contains the least number of virulence genes. n1 and n4 assays showed that MREC33 and HPM13 displayed the presence of *uidA*. n2 assay showed the presence of *eae* only in YSI16A and

Table 2. Multiplex PCR assays for determining the virulence factors.
bfpA in MREC33. n3 assay showed the presence of stx2 in YSI6A and MREC33. Isolate HPM13 showed the presence of ial. HPM13 showed the presence of aagR, revealed from n5 assay. None of the isolates showed the presence of five virulence genes elt, stla, stxl, stlb and aat.

3.9.5. Growth Kinetic Studies of the Test Strains in Presence of Nanoparticles

To quantify the antibacterial activity of the ZnO-PEI nanoparticles against MAR-HPI positive test strains, LD$_{50}$ of ZnO-PEI NP was determined. LD$_{50}$ values of ZnO-PEI for all the three tested strains were found to be 15 µg/ml. Growth was found to be completely arrested at 35 µg/ml of ZnO-PEI (Figure 5abc). This bactericidal activity of ZnO-PEI was

![Figure 5](image)

Figure 5. Growth curves of MAR-HPI positive isolates: (a) E.coli MREC33, (b) Klebsiella sp. YSI6A and (c) Shigella sp. MREC33 in liquid LB medium in presence of various concentrations of ZnO-PEI NP. Cultures were set up and grown under the same conditions, except that different concentrations of NPs were used (Δ, Control; ○, 10 µg/ml; △, 15 µg/ml; *, 25 µg/ml; and □, 35 µg/ml) and (d) % of viable MAR resistant bacterial cells in presence of ZnO-PEI and uncapped ZnO NP, both the nanoparticles having the same concentration (35 µg/ml).
compared with uncapped ZnO and the data indicate that former has much higher antimicrobial potential than later (Figure 5d).

3.9.6. Live/Dead Viability Assay
MREC33 cell viability was measured using Live/Dead BacLight Kit (Invitrogen). The Kit consists of two stains, propidium iodide (PI) and SYTO9. Green fluorescing SYTO9 is able to enter all cells irrespective of their nature and is used for assessing total cell count, whereas red fluorescing PI can only enter those cells which have compromised cytoplasmic membrane. It is usually assumed that bacterial permeability to nucleic acid dye such as propidium iodide (PI) is associated with the presence of substantial, irreparable breaches in the membrane, in the presence of which the organisms cannot maintain their membrane potential and are, therefore, nonviable. The intact cell (incubation for, 30 min) only shows green fluorescence (from SYTO9), as PI remains in the medium and does not fluoresce; in compromised cells (1.5 h), PI enters the cell and binds to DNA showing red fluorescence in addition to green fluorescence; in dead cells (2 h) the SYTO9 leaks out of the cell and thus only red fluorescence is visible (Figure 6).\textsuperscript{37} It was observed that the intensity of green fluorescence and red fluorescence (PI) of the analyzed cells changed as a function of increasing incubation time. For example, the

![Flow cytometric analysis of E. coli MREC33 in presence of ZnO-PEI nanoparticle using Live-Dead assay kit. After NP exposure cell samples were stained with mixture of SYTO9 and PI and analyzed on a cell sorter. 1 (= 0.5 h); 2 (= 1 h); 3 (= 1.5 h) and 4 (= 2 h) corresponds to incubation period. UL, UR, LL, LR in the figure stands for upper left, upper right, lower left and lower right.](image)

**Figure 6.** Flow cytometric analysis of *E. coli* MREC33 in presence of ZnO-PEI nanoparticle using Live-Dead assay kit. After NP exposure cell samples were stained with mixture of SYTO9 and PI and analyzed on a cell sorter. 1 (= 0.5 h); 2 (= 1 h); 3 (= 1.5 h) and 4 (= 2 h) corresponds to incubation period. UL, UR, LL, LR in the figure stands for upper left, upper right, lower left and lower right.
intensity of green fluorescence decreased at higher incubation time (2 h) indicating rapid loss of membrane integrity. The results further confirm that the ZnO-PEI nanoparticles affect the membrane integrity and also facilitate membrane lyses of cells. Internalization of ZnO-PEI in bacterial and mammalian cells was also compared and the data clearly showed that the NP internalization is much higher in case of bacteria, and this could be attributed to the presence of polyetheleneimine (PEI) at the surface of ZnO (discussed in chapter 4).

### 3.9.7. Towards an Understanding of the Mode of Action of ZnO Nanoparticle

#### 3.9.7.1. Morphological Transition and Cellular Damage

Effects of ZnO nanoparticles on MREC33 E. coli cell morphology were examined by scanning electron microscopy. After a 3 h treatment with 0.1 mg/ml of ZnO nanoparticles in LB broth under aerobic conditions, rod-shaped E. coli cells underwent a dramatic change in structural morphology. SEM studies clearly show that on NP exposure bacterial cells are damaged heavily with lysis occurring in cytoplasm membrane (Figure 7).

![Figure 7. SEM micrographs of (a) untreated bacterial cell and (b) cell in presence of ZnO-PEI (6 h of treatment).](image)

#### 3.9.7.2. Bacteriolytic Effect of ZnO-PEI Nanoparticle

In order to establish the mode of action of ZnO-PEI, it was further tested for protein leakage by cell lysis. The cell lysis was evidenced by protein profile of control and ZnO-PEI NP treated MREC33 cells. The relative leakage (%) was increased to 32.42% in the supernatant and decreased to 67.58% in the pellet of NP treated cells when compared to the pellet of control cells. The supernatant of control cells showed only 6.39% leakage (Figure 8a). SDS-PAGE also supported the results as indicated by decrease in the intensity of protein bands in NP treated pellet when compared to that of control pellet. In the earlier section SEM studies reveal ruptured of cell wall and cavity formation in MREC33 (Figure 8b). So this data further support our claim.
3.9.7.3. Measurement of Reactive Oxygen Species (ROS). Killing bacteria by generating ROS is the most common bactericidal mechanism of nanoparticles.\(^{38}\) Earlier, Lipovsky et al. detected hydroxyl radicals and singlet oxygen in the aqueous suspensions of ZnO NPs by EPR method and also demonstrated enhanced antibacterial activity of nanocrystalline ZnO attributed due to increased ROS-mediated cell injury.\(^{39}\) In a recent study they have elucidated the role of ROS mediated antifungal effect of ZnO NPs where ROS levels determined by EPR.\(^{35}\) In case of ZnO NP ROS can be generated through both extracellular and intracellular pathways.

3.9.7.3.1. Extracellular ROS Generation. Extracellular ROS was measured using XTT assay. The XTT tests have been shown to be consistent in the presence of quantum dots and to agree with spectroscopic measures.\(^{40}\) XTT measures radical generation colorimetrically when the generated radicals reduce the tetrazolium dye 2,3-Bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) to the highly colored (yellow) XTT formazan. It is found that ZnO-PEI generates more amount ROS compared to normal ZnO (Figure 9a). In presence of light the degree of ROS generation enhances several folds for ZnO-PEI (Figure 9b).

3.9.7.4.2. Intracellular ROS Generation

Intracellular ROS in the bacterial cells were measured using dichlorofluorescein diacetate (DCF-DA). In presence of ROS the dye gets oxidized and gives intense green...
fluorescence. It is found that ZnO-PEI induces a significant amount ROS inside the bacterial cell when treated at a concentration of 35 \( \mu \text{g/ml} \) (Figure 9c). To further validate ROS mediated bacterial killing, bacteria were grown in presence and absence of histidine. Histidine is a known scavenger of hydroxyl radicals, and we found that histidine (5 mM) inhibits the antibacterial effect of ZnO-PEI (Figure 9d).

![Figure 9](image)

**Figure 9.** (a) Extracellular ROS generation determined by XTT assay. (b) ROS generation by ZnO and ZnO-PEI in presence and absence of light. (c) Intracellular ROS generation determined by DCF-DA fluorescence. (d) The effect of histidine (5 mM) on growth curve of *E. coli* MREC33 in presence of ZnO-PEI (25 \( \mu \text{g/ml} \)).

### 3.9.7.5. DNA Fragmentation Analysis

The normal genomic DNA of MREC33 DNA was not fragmented whereas the ZnO-PEI nanoparticle (15 \( \mu \text{g/ml} \)) treated DNA shows fragmentation (Figure 10). In a separate

![Figure 10](image)

**Figure 10.** DNA fragmentation analysis; genomic DNA of *E. coli* MREC33 was treated with ZnO-PEI; lane 1 is control and lane 2 is ZnO-PEI NP treated.
study Kumar et al. has shown nuclear fragmentation on nanoparticle treated *E. coli* using confocal fluorescence microscopy, which further support our claim. ROS attacks cellular DNA and produce chain breaks, modification of carbohydrate parts, and nitro bases by oxidation, nitration, methylation, or deamination reactions. This lead to severe DNA fragmentation and ultimately cell death. Here, bacterial DNA damage starts occurring within the first two hours of nanoparticle treatment.

3.9.8. Synergistic or Additive Effect of ZnO-PEI Nanoparticle

We have screened different antibiotics + nanoparticle against various MDR bacteria, and we have found that tetracycline + NP combination is the most effective. To verify this claim we have performed bacterial growth curve with or without this formulation. Our result shows that when antibiotic or nanoparticle is added individually in the medium, the maximum killing is <50%, but when they are added in combination > 80% killing is achieved (Figure 11a). To further verify the above observation we have done zone of inhibition study with different antibiotics alone or in combination with the nanoparticle. We found that the maximum zone of inhibition occurs in case of tetracycline + NP combination followed by colistin sulphate (Figure 11bc and Table 3). We do not see any change in the zone of inhibition diameter when gentamycin or streptomycin were treated in combination with nanoparticle.

![Figure 11](image_url)

*Figure 11.* (a) Growth curve of *E. coli* MREC33 in liquid LB medium (○, Control; □, with 20 µg/ml tetracycline; Δ, 15 µg/ml ZnO-PEI NP; and ×, with both 15 µg/ml ZnO-PEI NP and 20 µg/ml tetracycline). Zone of inhibition (cm) of different antibiotics against test strain (*E. coli* MREC33) (b) in presence and (c) absence of ZnO-PEI NP (15 µg/ml per disk). In this figure A stand for ampicillin, Co stand for Co-trimoxazole, T stand for tetracycline, Sl stand for sulphatriad, S stand for streptomycin, G stand for gentamicin, Cl stand for colistin sulphate and Ch stand for cephalothin.
Table 3. Mean zone inhibition (mm) of different antibiotics (with or without ZnO-PEI) against E. coli MREC33

<table>
<thead>
<tr>
<th>Antibiotics name</th>
<th>Zone of inhibition (mm)</th>
<th>Zone of inhibition (NP + antibiotic) (mm)</th>
<th>Fold increase % = \frac{(b-a)}{a} \times 100^*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracyclin</td>
<td>0.8</td>
<td>1.1</td>
<td>37.5</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>1.4</td>
<td>1.4</td>
<td>No effect</td>
</tr>
<tr>
<td>Colistin Sulphate</td>
<td>1.6</td>
<td>1.9</td>
<td>18.75</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>1.5</td>
<td>1.5</td>
<td>No effect</td>
</tr>
</tbody>
</table>

*Percentage fold increases of individual antibiotics were calculated using the formula \( \frac{(b-a)}{a} \times 100 \), where \( b \) = zone of inhibition in presence of NP and antibiotic combination, and \( a \) = zone of inhibition in presence of antibiotic alone.

3.9.9. Effect of Protein Binding on Nanoparticles Mediated Killing

In this experiment we have used 35 μg/ml of ZnO-PEI NPs, where >95% inhibition was observed to establish the effect of BSA on nanoparticle mediated killing of bacterium. A fixed concentration of BSA was added to this media containing ZnO-PEI (Figure 12). We observe that in presence of BSA, % viability is approximately 28%, whereas in its absence the viability is approximately 2.6%. This suggests that the interaction between ZnO-PEI NP and BSA has an inhibiting role on the effect of the former in killing bacteria.

![Figure 12](image_url)

**Figure 12.** Interaction of BSA (35 mg/ml) with ZnO-PEI and its effect against antibacterial property of ZnO-PEI NP. Solid column represents % killed and the open column represents % of viability of MREC33 cells.

3.10. Discussion

Although many studies have reported the antibacterial activity of different NPs, the exact mechanism of nanoparticle toxicity is still uncertain. There are several questions that
need to be answered before any clinical utilization of nanoparticles. These are 1) whether
the toxicity of NPs are related to their size or it depends on their composition? Some
researchers have attributed NP’s cytotoxicity to their ability to generate ions.\textsuperscript{9,19}
However, there is no clear evidence available in literature to support this claim. 2) Dose
surface functionalization of nanoparticle play any significant role in antibacterial
activity? The general belief is that the surface functionalization has good impact on
antibacterial activity.\textsuperscript{41,42} Some investigators even have suggested that surface
functionalization on ZnO NPs have improved their antibacterial activity,\textsuperscript{15} which may be
attributed to enhanced ROS production by nanoparticle due to surface modification. 3)
Whether nanoparticle has any direct interaction with bacterial cell surface? If the answer
is affirmative, the question is how much deleterious is it? Bacterial outer membrane
disruption has been repeatedly observed and is considered to be the reason for NPs’
toxicity. But how the disruption happens is not clear. It may be caused by chemical
damage to membrane biomolecules, or may be caused by NPs robbing lipid molecules
through adsorption, or may be caused by membrane gelation or fluidization after NP
attachment.\textsuperscript{44} The effects of NP on outer membrane proteins is yet to be addressed
systematically.

Our experimental result indicates the involvement of two possible modes of
interaction between nanoparticles and bacteria: (1) direct interaction of PEI-capped NP
with lipopolysaccharide (LPS), which causes disruption and disorganization of bacterial
membrane and leakage of cytosolic content (Figure 7). We further monitored an
enhancement in nanoparticle internalization which may result from the possible loss of
membrane integrity. Our binding data also revealed that NP binding to bacterial surface is
fast, as we have found NPs inside the bacteria within couple of hours of treatment.
Bacterial membrane consists of very complicated architecture and the constituents are
very tightly packed inside the membrane. Incorporation of any foreign substance within
membrane architecture always leads to structural deformation and our case was not an
exception. We found an extensive membrane distortion and pore formation due to
bacteria-nanoparticle interaction. Once this pore formation occurs in the bacterial
membrane more amounts of antibiotic get access to bacterial cytosol and the organism
becomes more susceptible to antibiotic and as a result we witness a synergistic effect (Figure 11).

The increased antibacterial activity observed here is attributed to polyethyleneimine capping which enhanced the production of the reactive oxygen species (ROS) from ZnO (Figure 9). Reactive oxygen species (ROS), such as hydrogen peroxide (H$_2$O$_2$), superoxide anion (O$_2^-$), hydroxyl radicals (.OH) and organic hydroperoxides (OHPs) are toxic to the cells as they damage cellular constituents such as DNA, lipids, and proteins. The role of ROS in bacterial killing has been the subject of intense debate, and a general consensus seems to be elusive. In this investigation we have convincingly established that DNA damaged observed here is an outcome of ROS mediated oxidative stress. Further, we have shown that in presence of histidine (a known ROS quencher) the antimicrobial effect of ZnO is reduced several folds, which further confirms the role of ROS in ZnO mediated bacterial killing.

3.10.1. Toxicity of Nanoparticle Towards Eukaryotic Cells

Toxicity of NP always remains a controversial issue and its toxic dose varies depending on the nature of the cell line used. Recently Akhter et al. have shown that ZnO NPs have distinct effects on mammalian cell viability, killing cancer cells (HepG2, A549 and BEAS-2B), while posing no effect on normal cells (rat astrocytes and hepatocytes). The marked difference in cytotoxicity between cancer cells and normal cells suggests that ZnO mediated killing is maximum in fast proliferating cells. Fast proliferating cells produce more ROS in presence of NP compared to their slowly proliferating counterpart; so they are more prone to oxidative damage. Our result also shows a similar trend – among the three cell lines studied for toxicity, MCF-7 has the maximum proliferation potential (discussed in chapter 5), followed by PBMC and RBC. We also noticed that ZnO-PEI toxicity is maximum in case of MCF-7, followed by PBMC and RBC. Fortunately we found that ZnO-PEI concentration <25 mg/ml do not have any major cell destroying ability in RBC and PBMC cells and this concentration of NP has very good antimicrobial response against MDR bacterial strains. Considering the generation time of eutrophic bacteria and cancer cells, bacterial cell always possesses much faster generation time. Consequently, NP imparts more oxidative stress on bacteria. We have also been successful in applying combination therapy against MDR bacteria. It is because the
primary site of action of ZnO-PEI is bacterial membrane and the pore formed by NP makes an easy passage for the antibiotic.
3.11. References


