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Conjugation of enrofloxacin with amine functionalized zinc oxide nanoparticle enhances antibacterial activity *in vitro*

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Increased resistance to a large number of antibacterial drugs poses a serious challenge in chemotheraphy of infectious diseases. Here, we have made and attempt to redesign the existing chemotherapeutic agent enrofloxacin (EN) to treat resistant bacteria. Precisely, we synthezied EN conjugated zinc oxide nanoparticles (EN-ZNP) and explored enhancing the antibacterial activity of enrofloxacin. Zinc oxide nanoparticles (ZNP) were synthesized by microwave irradiation and amine functionalization by co-condensation with APTES and then by utilizing EPC/NHS chemistry, enrofoxacin was conjugated. Conjugation and their stability were confirmed by FT-IR spectra and Zeta potential. EN fraction in EN-ZNP was determined indirectly using UV-Vis spectroscopy. The MIC values obtained for EN-ZNP against MTCC cultures and clinical isolates of *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus* aureus were significantly (P <0.05) lower than ZNP and, when compared to native EN it is significantly higher. However, the concentration of conjugated EN in EN-ZNP was significantly lower than the MIC of native EN. The results suggest that enrofloxacin can be successfully conjugated with amine functionalized zinc oxide nanoparticles. The antibacterial efficacy was significantly improved when ZNP conjugated with EN against standard MTCC cultures and clinical isolates.

Keywords: Antibiotic resistance, Antimicrobial efficacy, Livestock, Nanomaterials, Multidrug resistance, Poultry

There is a widespread very high to extremely high multidrug resistance levels prevalent in many pathogenic bacteria for humans and animals to commonly used antimicrobial agents in member states of European Union[1]. Millions of cases and thousands of deaths occur each year due to antibiotic-resistant infections in the USA as well[2]. *Escherichia coli* and *Staphylococcus aureus* are the most common bacteria responsible for mastitis in bovines[3] and *Salmonella typhimurium* causes worldwide infections in humans and livestock[4]. The cell wall of gram negative bacteria (*E. Coli* and *Salmonella typhimurium*) consists of lipopolysaccharide (LPS) and gram positive bacteria (*Staphylococcus aureus* and *Streptococcus uberis*) consists of lipoteichoic acid (LTA), these induces release of variety of pro-inflammatory cytokines through activation of NFκB signalling pathway and continued production leads to multiple organ failure and deleterious effect on host[5].

Enrofloxacin (EN), the first fluroquinolone introduced into veterinary medicine[6], is still widely used for prevention and treatment of bacterial infections affecting livestock and poultry. However, indiscriminate use of EN has lead to development of resistant bacteria population with gene regulated alteration in efflux pump activity and biofilm formation[7] thereby reducing its clinical efficacy. Treatment with EN is reported to induce highest number of mutations at *arc* gene and *glpF* alleles, and plays an important role in persistence, leading to chronicity of the disease[8]. Increased minimum inhibitory concentration (MIC) and more number of resistance genes (*tetK*, *tetM* and *blaZ*) and virulence genes (*hla*, *fnbA*, *fnbB*, *eta*, *etb*, *sea*, *sec*, *she* and *seJ*) contribute to permanence of bacteria in the mammary gland resulting in persistence mastitis[9]. Earlier study found that multidrug-resistant commensal *E. coli* isolates were selected in the gut of poultry when EN is used to treat some other bacterial infections in chicken[10]. EN along with LPS exacerbates cellular toxicity by initiating the production of reactive oxygen species (ROS) and reduction of cellular antioxidant enzymes and an increase of lipid peroxidation in tissues[5].

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Lately, nanoscale materials like inorganic metal and metal oxide nonoparticles, such as Ag, Cu, CuO, ZnO, TiO_2, Fe_3O_4 and Mg have emerged as novel antimicrobial agents owing to their excellent physicochemical properties, such as higher aspect ratio and large surface area to volume[11]. Zinc oxide nanoparticles (ZNP) have antibacterial, antifungal, anticancer, anti-inflammatory and antidiabetic properties owing to the semiconductor properties of ZnO that generates ROS[12]. In addition, ZNPs induce changes in gene expression and subsequent downregulation or upregulation of respective amino acids and pyrimidine biosynthesis in *S. aureus*[13].

Zinc oxide (ZnO) is a very fine, odourless, amorphous white powder[14] and US FDA has recognized bulk ZnO as a 'Generally Recognized As Safe (GRAS)' substance[12]. Zinc is an important cofactor in various cellular mechanisms and plays an important role in maintaining cellular homeostasis in mammalian tissues. Hence, ZnO shows high biocompatibility[11] and ZnO nanoparticles are used in veterinary therapeutics without much side effects or residues in edible animal food products. Furthermore, nanomaterial can also act as potential carriers for delivery of different synthetic drugs and natural products[15] and, ZnO NPs are excellent drug carriers[12]. Conjugation of various nanoparticles with natural extracts[15] and antibiotics[16] enhances the antimicrobial activity, especially against bacteria. In this context, the present study has been designed to synthesize and characterize enrofloxacin conjugated zinc oxide nanoparticles (EN-ZNP), and evaluate its antibacterial effect against MTCC cultures and clinical isolates of various critical infection causing bacterial strains *in vitro*.

Materials and Methods

Chemicals and Media

All the chemicals and microbial culture medias are analytical grade and obtained from various commercial sources (Sigma-Aldrich, Loba Chemie, Thermo fisher Scientific (India) Pvt Ltd, Hi-Media Ltd). Pure enrofloxacin (99%) was supplied as gratis from INTAS Pharma, Pvt, Ltd, Mumbai.

MTCC reference microbial cultures

Eschericha coli, *Salmonella typhimurium* and *Staphylococcus aureus* were procured from MTCC, CSIR-Institute of Microbial Technology (IMTECH), Chandigarh, India.

Clinical isolates

Clinical isolates (*E. coli*, *Salmonella* spp. and *Staphylococcus* spp.) were obtained from the departments of Veterinary Microbiology and Veterinary Public Health from various constitutive veterinary colleges of Sri Venkateswara Veterinary University.

Synthesis of Zinc Oxide Nanoparticles

Zinc oxide nano particles (ZNP) were synthesized using zinc acetate and tris (hydroxymethyl) aminomethane (TRIS) buffer as per the procedure described by Patra *et al*.[17]. Briefly, 20 mL of 20% aqueous TRIS solution was added drop by drop to 25 mL of 0.05M zinc acetate dihydrate solution while stirring with magnetic stirrer at room temperature (25ºC). The mixture was further stirred well and then subjected to microwave irradiation (300 Watt for 3 min). The product thus obtained was centrifuged at 10000 rpm for 10 min and washed several times with triple distilled water to remove excess of TRIS buffer. Finally, the product was washed with ethanol and dried overnight at 80ºC in a hot air oven to obtain ZnO nanoparticles.

Amine functionalization of ZnO Nanoparticles

Amine functionalization of ZNP was carried out by co-condensation method as reported by Patra *et al*.[17]. Briefly, 0.5 g of ZNP was dispersed in 50 mL dimethyl sulfoxide (DMSO) in a sonication bath for 1 h. The resultant dispersion was transferred to a round bottom flask attached to a reflux condenser and 400 µL of 3-aminopropyltriethoxysilane (APTES) was added. The solution was refluxed at 120ºC for 3 h. After completion of the reaction the resulting nanoparticle was centrifuged at 12000 rpm for 15 min and washed several times with ethanol to remove the unreacted APTES. Finally, the product was dried at 60ºC overnight to produce amine functionalized ZNP (AF-ZNP).

Amine functionalization of ZNP was confirmed using the Ninhydrin test[18]. Ten milligrams of amine functionalized ZNP was suspended in 1.0 mL of distilled water and to this solution a few drops of ninhydrin reagent was added and vortexed. The test tube was placed in a boiling water bath for 5 min and cooled to room temperature. Successful amine functionalization was confirmed by the development of deep blue or purple (Ruhemann's purple) colour.

Conjugation of Enrofloxacin with ZnO nanoparticles

Enrofloxacin (EN) was chemically conjugated with amine functionalized ZNP using 3-ethyl

dimethylaminopropyl carbodiimide/N-hydroxy succinimide (EDC/NHS) chemistry as per the procedure described by Patra *et al*.[17]. Briefly, 15 mg of EN was dispersed in 30 mL of 1:1 aqueous DMSO by sonication; to this 8 mg of EDC was added followed by addition of 8 mg of NHS while maintaining the pH of the solution at 6.0. The resultant mixture was stirred for 3 h in darkness for activation. After activation of EN, 30 mg of amine functionalized ZNP dispersed in 10 mL of 1:1 aqueous DMSO was added drop wise. The pH was maintained at 8.0 and the reaction mixture was stirred overnight. The reaction mixture was centrifuged at 4000 rpm for 15 min followed by repeated washings with DMSO and ethanol to obtain EN-ZNP. The EN-ZNP obtained was finally dried at 60°C and stored in a cool and dry place.

Determination of Enrofloxacin concentration in EN-ZNP

The conjugated fraction of EN was determined indirectly by finding the unconjugated fraction in the supernatant and washings from the conjugation process using UV-Vis spectrophotometry against standard enrofloxacin concentrations[19].

Conjugation efficacy

The efficacy of enrofloxacin conjugation with amine functionalized ZNP was determined by using the following formula:

$$\text{Conjugation efficacy (mg\%)} = \frac{\text{weight of Enrofloxacin used (mg)} - \text{unconjugated Enrofloxacin (mg)}}{\text{weight of Enrofloxacin used (mg)} + \text{weight of amine functionalized ZNP (mg)}} \times 100$$

Characterization of ZNP and EN coated zinc nanoparticles (EN-ZNP)

UV-Visible spectra analysis

The UV-Visible spectrum of all the three components EN-ZNP, ZNP and EN was observed by UV-Vis Spectroscopy (UV-2450 Shimadzu) at the wavelength of 250-650 nm[20] by mixing a small aliquot with pure Ethanol.

Fourier Transform Infrared (FT-IR) *spectroscopy analysis*

FT-IR spectra analysis of ZNP, amine functionalized ZNP (ZNP-AF) and, EN-ZNP on TENSOR-27 (BRUCKER) in the diffuse reflectance mode operated at a resolution of 4 cm^{-1} in the range of 4000 to 400 cm^{-1} was carried out to evaluate the Zn-O bond, other molecules and functional groups that might be involved in the conjugation of nanoparticles and their stabilization[20]. FT-IR analysis was carried out at Chalapathi Institute of Pharmaceutical Sciences, Guntur, Andhra Pradesh.

Transmission Electron Microscopy *(TEM)*

The morphology and size of the ZnO nanoparticles were studied by transmission electron microscopy (JEOL-JEM-1010 instrument) with an accelerating voltage of 80 kV as described by Ahmad *et al*.[21] (2015). A well dispersed sample of ZNPs was coated on carbon-coated-copper TEM grids under vacuum in a desiccator and loaded into the specimen holder. The particle size and surface morphology of the nanoparticles was evaluated using ImageJ 1.45s software (Department of Chemistry, Indian Institute of Technology, Madras).

Dynamic Light Scattering (DLS)

Dynamic Light Scattering (DLS) technique was used to determine the hydrodynamic radius or size of ZNP in suspension using the instrument HORIBA Scientific Nano Partica (SZ-100)[22] at Frontier Technology Laboratory, Regional Agricultural Research Station, Tirupati.

Zeta potential

Zeta potential study[23] was carried out to detect the surface charge and stability of the prepared ZNP by using the instrument HORIBA Scientific Nano Partica (SZ-100) in zeta mode with an electrode voltage of 3.4 V at 25ºC. The measurement was carried out at Frontier Technology Laboratory, Regional Agricultural Research Station, Tirupati.

Antibacterial activity of EN-ZNP

Minimum inhibitory concentration of EN-ZNP

The antimicrobial activity of various synthesized compounds ZNP, EN and EN-ZNP was evaluated by micro dilution method as per Clinical and Laboratory Standards Institute (CLSI 2006)[24]. Stock solution of EN-ZNP was prepared by suspending 10 mg of EN-ZNP in 10 mL of nutrient broth. A two fold dilution of this solution was made in 100 µL of Mueller-Hinton (MH) broth in a microplate. To each well, 50 µL of 1:10 diluted 0.5 McFarland units of bacterial suspension was added to provide a final concentration of 5×10^5 cfu/mL per well. Positive and negative controls for culture and broth were maintained. The plates were incubated at 37ºC for 18 h. One hour before the completion of incubation, 50 µL of nitro blue tetrazolium chloride (NBT) (2 mg/mL in distilled water)[25] was added to each well and the plates were incubated at 37ºC for another hour. The MIC was defined as the minimum concentration of the compound, which inhibited visible growth of bacteria, evidenced by the lack of development of any colour.

Statistical analysis

The antibacterial activity of MTCC Cultures and their clinical isolates was represented by the determination of Minimum Inhibitory Concentration (MIC) with their respective standard error. Statistical package for social sciences (IBM SPSS 19.0 version) was used for comparing the means of MIC using t-test and one way ANOVA. The level of significance was set at 0.05.

Results

As illustrated in Fig. 1, ZNPs were synthesized by microwave irradiation of zinc acetate in strong alkali condition. Amine functionalized group on ZNP was added by co-condensation reaction using APTES. Then amine functionalized ZNP was conjugated to EN at carboxylic group using EDC/NHS chemistry.

Physicochemical properties

The TEM image of ZNP revealed small and spherical shape with average size of approximately 20 nm (Fig. 2A). However, in most occasions, agglomeration of particles was observed with agglomerated nanoparticles size around 50 nm. Dynamic light scattering analysis of ZNP showed a hydrodynamic diameter (HDD) of 28.3 nm (Fig. 2B) which is almost similar to TEM measurement. The estimated zeta potential of ZNP was –29.7 mV at pH 7 (Fig. 2C).

Analysis of UV-Visible spectra of all the three components ZNPs, EN and EN-ZNP revealed chemical conjugation of enrofloxacin with ZNP (Fig. 3). ZNP exhibited a steep peak at 380 nm, native EN exhibited two characteristic peaks, one at 280 nm and other at 321 nm. After conjugation of EN with ZNP, the first peak did not shift. However, the second peak shifted slightly to 375 nm corresponding to ZNP.

The functional groups involved in, successful conjugation and stabilization of EN-ZNP was assessed by FT-IR spectra analysis of AF-ZNP, EN and EN-ZNP (Fig. 4). Amine functionalized ZNP showed transmission peaks at 3858.67, 3741.64, 3307.29, 2929.53, 2361.18, 1573.32, 1513.15, 1385.95, 1110.87, 1012.61, 570.45, 455.32 and 416.93cm^{-1}. Enrofloxacin showed peaks at 3859.34,

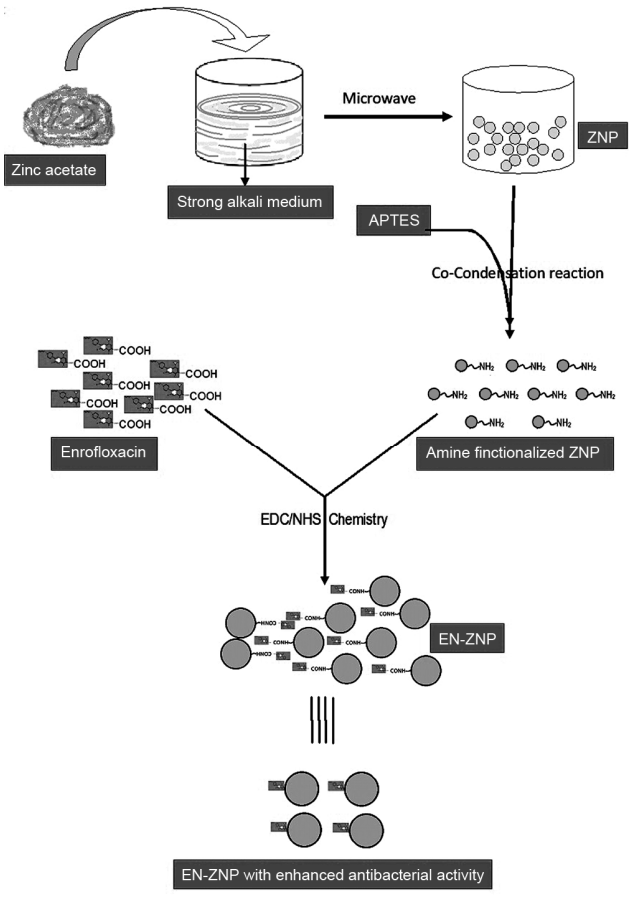

Fig. 1 — Schematic representation illustrating the whole process of chemical conjugation of enrofloxacin

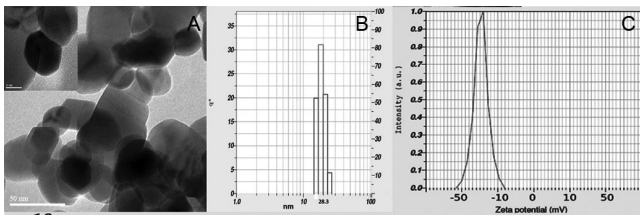

Fig. 2 — (A) TEM image of ZNP; (B) DLS analysis of ZNP showing hydrodynamic radius of 28.3 nm; and (C) Zeta potential of ZNP (-29.7 mv)

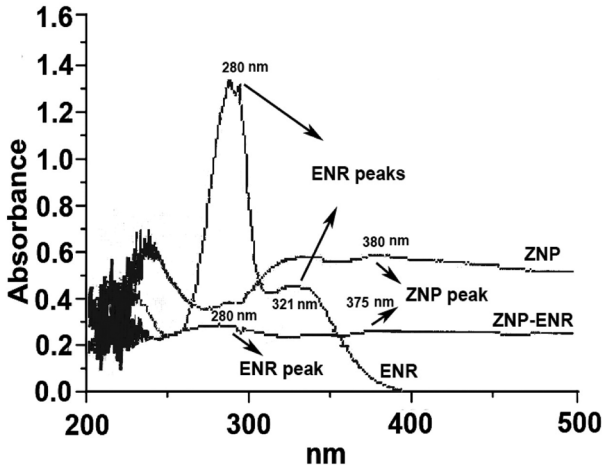

Fig. 3 — UV-Vis spectrum of enrofloxacin and all three components of ZNP

3740.85, 3086.06, 2967.24, 2873.38, 2824.81, 2351.82, 1901.54, 1847.37, 1735.53, 1626.18, 1461.78, 1387.67, 1253.48, 1151.12, 1047.96, 1017.35, 943.30, 887.75, 830.96, 791.25, 746.26, 702.38, 630.85, 554.83, 483.28 and 421.49 cm^{-1}. Enrofloxacin conjugated ZNP showed peaks at 3837.88, 3741.78, 3380.79, 2927.04, 2361.37, 1628.34, 1559.83, 1488.17, 1396.88, 1305.29, 1257.35, 1121.02, 1023.59, 896.47, 566.60 and 422.93 cm^{-1}.

Enrofloxacin conc. in EN-ZNP

The EN concentration in EN-ZNP was 1.18 mg%, based on this the actual concentration of enrofloxacin present in EN-ZNP was calculated and showed in parenthesis to MIC of EN-ZNP against various microorganisms (Table 1).

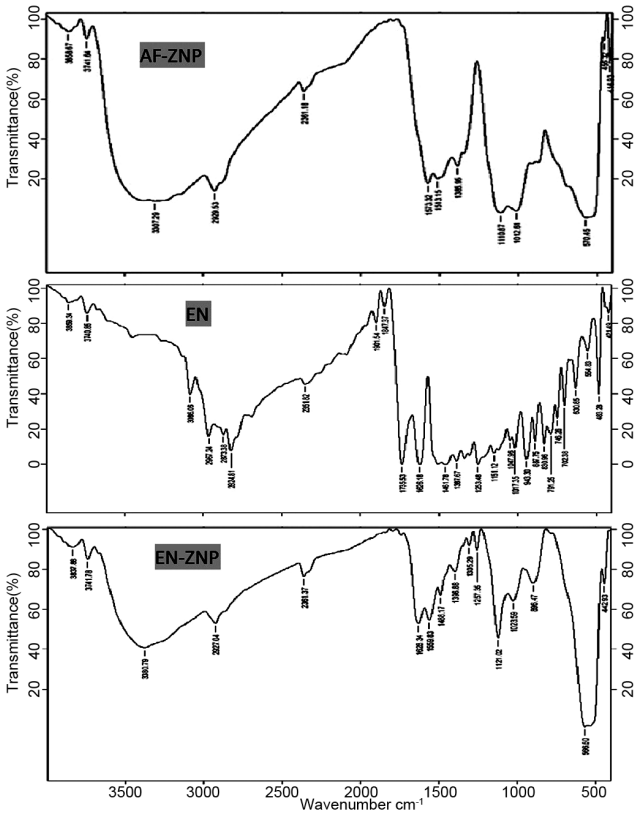

Fig. 4 — FT-IR spectra of amine functionalized ZNP (AF-ZNP), enrofloxacin (EN) and Enrofloxacin conjugated ZNP (EN-ZNP).

Antibacterial activity of EN-ZNPs

The antibacterial activity of EN-ZNP was evaluated against both standard MTCC cultures and clinical isolates (Table 1) and compared with native ZNPs and native EN.

Against standard MTCC cultures

The MIC (μg mL^{-1}) of native enrofloxacin was 0.047±0.001 for *E. coli*, 0.032±0.007 for *S. typhimurium* and 0.106±0.019 for *S. aureus*. Native ZNPs showed an MIC (μg mL^{-1}) of 58.530±0.030 for *E. coli*, 49.120±0.032 for *S. typhimurium* and 56.020±0.060 for *S. aureus* that was significantly (P <0.05) reduced when conjugated with enrofloxacin that is EN-ZNP.

The MIC of EN-ZNP along with actual enrofloxacin (present in conjugated form) concentration in brackets (μg mL^{-1}) was 1.588±0.0000 (0.0187±0.0043) against *E. coli*, 0.438±0.024 (0.0051±0.0015) against *S. typhimurium* and, 5.401±0.849 (0.0637±0.0078) against *S. aureus*. Although the MIC (μg mL^{-1}) values of enrofloxacin were significantly (P <0.05) lower for each organism compared to either native ZNP or EN-ZNP, the MIC of actual concentration of conjugated enrofloxacin in EN-ZNP was significantly (P <0.05) lower than native EN for respective MTCC cultures.

Against clinical isolates

Enrofloxacin conjugated ZNP was tested on clinical isolates of *E. coli* (12 samples), *Salmonella* spp. (6 samples) and *S. aureus* (10 samples). Two isolates of *E. coli* were found to be resistant to EN with a MIC >0.125 μg mL^{-1}. Similarly, one isolate of *Salmonella* and two isolates of *S. aureus* were found to be resistant to EN. Remaining isolates were susceptible to native EN with an average MIC (μg mL^{-1}) of 0.0975±0.0161 (*E. coli*), 0.079±0.0154

Table 1 — Minimum inhibitory concentration (μg mL^{-1}) against standard MTCC and clinical isolate cultures

Culture	Enrofloxacin (μg mL^{-1})	ZNP (μg mL^{-1})	EN-ZNP (μg mL^{-1})	Enrofloxacin concentration in EN-ZNP (μg mL^{-1})
Escherichia coli (MTCC 443)	0.047±0.001^{b}	58.530±0.030^{d}	1.588±0.000^{c}	0.0187±0.0043^{a}
Salmonella typhimurium (MTCC 3224)	0.032±0.007^{b}	49.120±0.032^{d}	0.438±0.024^{c}	0.0051±0.0015^{a}
Staphylococcus aureus (MTCC 3160)	0.106±0.019^{b}	56.020±0.060^{d}	5.401±0.849^{c}	0.0637±0.0078^{a}
E. coli (clinical isolate)	0.0975±0.0161^{b}	99.034±8.753^{d}	6.941±0.589^{c}	0.083±0.0019^{a}
Salmonella typhimurium (clinical isolate)	0.079±0.0154^{b}	81.481±8.189^{d}	5.787±1.180^{c}	0.069±0.002^{a}
Staphylococcus aureus (clinical isolate)	0.111±0.032^{b}	80.462±12.05^{d}	7.222±0.648^{c}	0.086±0.001^{a}

[Values are Mean ± Standard Error (MTCC cultures: n = 3; Clinical isolates: *E. coli*: n = 12, *S.typhimurium*: n = 6 and *S. aureus:* n = 10). Horizontal means with different superscripts are significantly different (P <0.05). One way ANOVA followed by Tukey's *post hoc* test using Statistical Package for Social Sciences 19.0 version. ZNP: Zinc oxide nanoparticles; EN-ZNP: Enrofloxacin conjugated zinc oxide nanoparticles]

(*Salmonella* spp.) and 0.111±0.032 (*S. aureus*). The average MIC of native ZNP (µg mL^{-1}) was 99.034±8.753 against *E. coli*, 81.481±8.189 against *Salmonella* spp. and 80.462±12.050 against *S. aureus*. The average MIC of EN-ZNP along with actual enrofloxacin concentration in parenthesis (µg mL^{-1}) for clinical isolates was 6.941±0.589 (0.083±0.0019) against *E. coli*, 5.787±1.180 (0.069±0.002) against *Salmonella* spp. and 7.222±0.648 (0.086±0.001) against *S. aureus*, which is significantly lower as compared to MIC of ZNP against respective isolates. The MIC of actual enrofloxacin in the EN-ZNP was comparable to native EN and concentrations of enrofloxacin present in EN-ZNP were shown in parenthesis of respective MIC expressed above.

Discussion

The discovery of new antibiotics becomes critical as more antibiotics are rendered ineffective by drug resistant strains of microorganisms, therefore shifting the focus towards finding alternatives to antibiotic therapies for infections[26]. Recent advances in the field of nanotechnology, especially metal oxide nanoparticles, possess some advantages, such as high stability, simple preparation process; ability to be prepared in desired size, shape and easy to add functionalization groups on the surface, thereby, holds great potential for development of new antibacterial agents for fighting multidrug-resistant microorganisms that could substitute antibiotics[27]. ZnO nanoparticles, with their novel physicochemical properties, are nontoxic, chemically stable, biocompatible, antidiabetic, anticancerous, drug carriers, and showed antimicrobial activity against *E. coli*, *S. aureus* and *S. typhi*[28] along with an ease of functionalization making it highly feasible for the development of antibacterial formulations[29].

Enrofloxacin conjugated ZnO nanoparticles (EN-ZNPs) synthesized by microwave irradiation on zinc acetate in strong alkali medium and TEM analysis revealed almost spherical shape and 20 nm of size that is similar to earlier findings of spherical and 25-30 nm size[30]. However, there is little agglomeration of ZNPs with size of 50 nm. Hydrodynamic diameter of ZNPs was 28.3 nm which is almost similar to TEM measurement. The estimated zeta potential of ZNPs was –29.7 mV at pH 7, which is almost similar to the previous findings of 28.2 mV[20] which suggests that capping molecules on surface of ZNPs are negatively charged groups and have good stability[30] in colloidal solutions.

Then ZNPs were amine functionalized by co-condensation with APTES and then by utilizing EDC/NHS chemistry, enrofloxacin was conjugated. Initial UV-Vis spectra analysis (Fig. 3) of ZNPs exhibited steep peak at 380 nm which is in conformity with the previous study[28] range of 360-380 nm of light absorption. Enrofloxacin exhibited two characteristic peaks, one at 280 nm and other at 321 nm. After conjugation of EN with ZNPs, the first peak did not shift, however, the second peak shifted slightly to 375 nm corresponding to ZNPs that is characteristic of EN conjugation to ZNPs as mentioned by other researchers[17].

FT-IR spectra analysis carried out to detect the presence of Zn-O bond and EN in synthesized product to confirm the conjugation of EN with AF-ZNP in EN-ZNP. In FT-IR spectra (Fig. 4) amine functionalized ZNPs showed a characteristic peak at 3307.29 cm^{-1} due to the presence of O–H stretching frequency along with –N–H stretching frequency of NH2 group. A small distinct peak at 1385.95 cm^{-1} signified –O–H bending with a lower intensity, which was also most similar to Patra *et al*.[17] observations. The FT-IR spectra of EN have two characteristic absorption peaks, 1735.53 cm^{-1} and 1626.58 cm^{-1}; the first is the C=O vibration absorption peak from carboxylic acid oxygen, and the second was from keto C=O peak from the ring of EN and small peaks obtained in the range of 1250 cm^{-1} to 1500 cm^{-1} are characteristics of enrofloxacin. Similar observations were made by Patra *et al*.[17] where they stated that, characteristic peaks for ciprofloxacin lies between 1400 cm^{-1} to 1550 cm^{-1}. The FTIR spectra of EN-ZNP showed peaks obtained at 3380.79 cm^{-1}, 2927.04 cm^{-1} justifying O–H and C–H stretching, respectively. However, the 1735.53 cm^{-1} peak of EN disappeared in the complex and; instead two very characteristic bands were present around 1628.34 cm^{-1} and 1559.83 cm^{-1} that could be amide doublet[31] and the small peaks obtained in the region of 1250 cm^{-1} to 1500 cm^{-1} were similar to enrofloxacin. Our FT-IR spectra results are in correlation with Alamdare *et al*.[30] who stated that any shift or change in the position and intensity of peaks in the sample spectrum can be correlated with the interaction of functional groups.

The *in vitro* antimicrobial efficacy of EN-ZNP on standard cultures and clinical isolates was found to be dose-dependent. Partial growth of various bacterial strains was visible up to 20 (0.236) µg/mL of EN-

ZNP concentration, while no growths was observed above 20 (0.236) µg/mL of EN-ZNP concentration. The MIC values for EN-ZNP against various MTCC cultures ranged from 0.438±0.024 (0.0051±0.0015) (*S. typhimurium*) to 12.037±0.463 (0.1444±0.0009) (*P. aeruginosa*) µg/mL. The MIC values of EN-ZNP against various MTCC cultures were significantly lower than ZNP and when compared to native EN it is significantly higher, however the concentration of conjugated EN in EN-ZNP was significantly lower than the MIC of native EN. Meanwhile the susceptibility of clinical isolates is highly variable to EN-ZNP and native EN and ZNP (Table 1). Few clinical isolates of *E. coli*, *Salmonella* spp. and *S. aureus* were resistant to native EN and their MIC of EN-ZNP was significantly lower than ZNP and when compared to EN it is significantly higher. However when compared with the amount of EN that is conjugated in EN-ZNP it was significantly lower than native EN. When the average values are compared the MIC values of EN-ZNP are significantly lower than ZNP and MIC of EN that is present in EN-ZNP was significantly lower than MIC of native EN.

The higher *in vitro* antimicrobial efficacy of EN-ZNP against MTCC cultures and clinical isolates could be attributed to the combined effect of ZnO and EN on bacteria. Excellent physicochemical properties like large aspect ratio, surface area[29] enables the ZNPs to bind effectively to the bacterial cell membrane and because of semiconductor property[12] it generates ROS thereby disintegrate the cell membranes. After mechanical damage to cell membrane, ZNP enters into the cytoplasm where it interacts with biomolecules causing oxidative stress and bacterial cell death[32]. Even though the less quantity of EN present in EN-ZNP is effectively inhibiting the microorganisms because of ZNP induced damage to bilipid layers and oxidative stress and easily reaches to the site of action and inhibits bacterial growth. Previous studies showed that ZNP induces morphological defects on surface[33] that could contribute to the mechanical damage of the cell membrane of the bacteria[34] and oxidative stress playing important roles in the antibacterial activity[35,36].

Over all, the ZNP conjugation with enrofloxacin (EN) has proved the antibacterial efficacy of EN against test strains even at sub-MIC concentration. It suggests that only lower amounts of EN is required to produce the same antibacterial effect as native EN and also overcomes the development of bacterial resistance due to gene regulated alteration in efflux pump activity and biofilm formation[7] against EN. It can be speculated that the penetration of enrofloxacin into the cells may be accelerated in combination with ZNP, which results in more interactions with target sites that are important for the antibacterial activity of enrofloxacin.

Conclusion

The above findings demonstrate that enrofloxacin (EN) forms a stable complex with zinc oxide nanoparticles (ZNP). Additionally, EN specifically binds on the surface of ZNP to enhance antibacterial activities of both compounds against different isolates of *E. coli*, *Salmonella* spp. and *S. aureus*. EN-ZNP can be a suitable new therapeutic entity to treat bacterial infections of poultry and live stock without the problems of bacterial resistance development and residues in edible animal products. Further research is necessary to determine the antibacterial effect of enrofloxacin conjugated ZNP in other bacterial test strains and *in vivo* antibacterial efficacy and toxicity studies.

Conflict of Interest

Authors declare no competing interests.

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