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Synthesis of iron nanoparticles, its characterization and anti-microbial activity assessment

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We reported the efficacy of the Iron nanoparticles (IONPs) and assessed two different approaches for the synthesis of IONPs *i.e.* Polyol and co-precipitation method and further, evaluate their antimicrobial properties. Ferrous sulphate heptahydrate salts were reduced with ethylene glycol to obtain IONP and Fe^{+2} and Fe^{+3} iron precursor salts were used for co-precipitation reaction that was performed with KOH at optimum heating. Further, synthesized (IONPs) were characterized by hydrodynamic radii measurement done by DLS clearly indicating the size of IONPs is 79.75 nm in polyol based and 135.1 nm in co-precipitation method. The biological efficacy in terms of antimicrobial activity was assessed by the Kirby Bauer method, applied for both Gram-positive and Gram-negative bacteria such as *Staphylococcus aureus* and *Escherichia coli*, respectively. The ZOI values *i.e.* Zone of inhibition diameter was found to be clearly visible in both *S. aureus* and *E. coli*, indicating bactericidal activity. Further growth kinetics studies and bacterial genotoxicity was also assessed. Hence, IONPs synthesized are proposed to have great potential as an antibacterial agent and can be used in drug delivery.

Keyword: Bacterial genotoxicity, Co-precipitation method, Growth kinetics, Kirby-Bauer method, Polyol method, Zone of inhibition

Bacterial resistance magnification against existing antibiotics remained a major health challenges worldwide. The production of slime by some bacterial strains facilitates the formation of biofilms on any surface which enhances bacterial resistance by inhibiting the antibiotic action of certain drugs. The most common interactions like H-bond, Vander Waals, electrostatic, dipole-dipole and hydrophobic mediate the adhesion of bacteria on any surface. Various nanoparticles possess antimicrobial activity which can inhibit the formation of biofilm as well as bacterial adhesion by specific interaction. When nanoparticles are dispersed in biological based medium could interact with the various biological interface such as protein, lipids, polysaccharides, and DNA either by specific or non-specific interaction[1-2]. Hence, understanding these interactions at Nano-biointerface could help in designing safe, novel, and more efficient therapeutic nanoparticles. Nanoparticles (NPs) are emerging as the key players in nanotechnology development. NPs comprise nano-scale size in the nanometer range (1-1000 nm) having a large surface area to volume ratio. Because atoms are distributed on the surface of nanoparticles offers them a vast area of physical, chemical, and biological properties in various fields like drug and gene delivery, bio-nanotechnology, nanomedicine, biosensors, *etc*. NPs can be categorized on the basis of size, morphology, and chemical and physical properties and can be broadly classified as organic and inorganic groups. Organic groups consist of polymeric and synthetic nanoparticles and inorganic groups such as Carbon nanotubes (CNTs), fullerenes, and metallic/metallic oxides nanoparticles like gold (AuNPs). Silver (AgNPs), Zinc oxide (ZnO NPs). Naturally, iron (III) oxide is present in rust form and known to have much potential that can be used in biomedicine, environment, and agriculture. Three forms of iron oxides are found in nature magnetite (Fe_3O_4), maghemite (γ- Fe_2O_3), and hematite (α- Fe_3O_4). These nanoparticles are especially used in the biomedical application for protein immobilization like diagnostic magnetic resonance imaging (MRI) and drug delivery. The majority of studies include iron oxide nanoparticles (IONPs) that have a round shape which eliminate it significant contribution to the antimicrobial action. Therefore, we evaluated the effect of sizes and compositions of IONPs on its antibacterial activity[3].

Materials and Methods

Materials

For chemical preparation of iron nanoparticles, different salts such as ferrous sulphate heptahydrate

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(FeSO4.7H2O, 99.0% pure), (FeCl3.6H2O, 99.0% pure) are acquired from Sigma Aldrich. Potassium hydroxide (KOH), Sodium hydroxide (NaOH) as well as polyol solvent *i.e.* ethylene glycol was provided by SRL Pvt. Ltd., India Deionized water.

For antibacterial tests, different bacterial model test strains such as for gram negative bacteria *E. coli* strain (MTCC-1677) and gram positive bacteria *S. aureus* strain (MTCC-3160) was used. These bacterial strains were obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India. Culture media such as bacterial specific broth, agar, antibiotic (Gentamycin) were purchase from company Hi-Media. Deionized water was used throughout the experiment.

Synthesis of iron nanoparticles

Synthesis of iron nanoparticles using polyol method

Synthesis of iron nanoparticles was done with ethylene glycol as solvent agent. This solvent is also known for its reducing properties thus act as a weak reducing agent, synthesis was performed as per protocol specified by M. Abbas *et al*. It involves the addition of 60 mg of iron precursor salt *i.e.* FeSO4.7H2O to a solvent solution here represented as ethylene glycol that was kept on heat to an approaching boiling temperature of 180°C. To this NaOH was also poured dropwise, its presence is known to accelerate the rate of slow reducing reaction. The synthesis reaction was continued until the greyish black color was obtained, indicating the formation of iron nanoparticles. The washing step was performed with ethanol: water mix. After this nanoparticles were left in vacuum oven for drying[4].

Synthesis of iron nanoparticles using co-precipitation method

Iron nanoparticles were synthesized with iron^{+2} and iron^{+3} salt added in the ratio of 1:2 in deionized water. The reaction was started with initial heating of the solvent at 80°C in which 3.0 mol/L of KOH solution was also added dropwise, to accelerate the reaction rate. Let the blackish-greycolor appear. Allow the reaction to cool. Separate and wash the iron nanoparticles with a mixture of ethanol and water. Dry in oven at 100°C[5].

Characterization of the nanoparticles

The nanoparticles were further characterized for structural, properties such as Size, surface charge *etc*. Size and surface zeta potential was determined with Zetasizer, Malvern analytical, which is based on working principle of dynamic light scattering also known as DLS.

Antibacterial tests

Preparation of nutrient broth

The sterilized nutrient broth was prepared based on the instruction performed stepwise on nutrient broth manufacturer's bottle. This was further autoclaved at a set temperature of 120°C, 15 psi for duration of 20-30 min. Further mother inoculums was inoculated in ratio of 1:100 in this sterile broth which was kept in an incubator (orbital shaker) at temperature of 37°C for 24 h. Measuring turbidity as absorbance value at 600 nm using UV spectrophotometer (Cary 60, Agilent Technologies) was performed and colony forming unit per mL also illustrated as (CFU/mL) was also calculated as:

$$1OD = 0.8 \times 10^9 \text{ CFU/mL}$$

Antibacterial activity using disc diffusion assay

Antibacterial activity of nanoparticles was determined with the Kirby Bauer technique. Bacterial culture was suspended in soft agar and was spread evenly onto the nutrient agar plate that was initially prepared. Sterilized paper discs of diameter 5 mm were soaked with test samples *i.e.* different type of iron NPs at different conc., based on the synthesis approach, antibiotic gentamycin is used as positive control along with sterile Milli-Q water as negative control was positioned on the plate, kept for incubation at temperature of 37°C and ZOI measurements were recorded post 24 h[6-7].

Growth kinetic studies

Sterile broth was initially prepared and was inoculated with bacterial culture. Two different test samples were prepared by dissolving 1 mL of LB, 10 µL of bacteria cultures, 20 µL of different iron nanoparticles (conc.10 mg/mL) were prepared, and sterile LB media with bacterial culture inoculums was considered as control. Absorbance values were measured at 600 nm at different time points and data is represented as log CFU/mL as a function of time for both the experimental/control groups to plot the growth curves[8-9].

Quantitative estimation of fragmented DNA using Diphenylamine (DPA) assay

Fragmentation of bacterial genome was evaluated. Freshly prepared bacterial cultures were utilized in the assay and treatment was given to the synthesized nanoparticles at concentrations (10 mg/mL) for 24 h. Post-treatment bacterial cell pellet was collected at 300 g for duration of 10 min at 4°C temperature, the supernatant was discarded, mix it further with 800 µL

of PBS buffer, and 700 µL freshly prepared ice-cold lysis buffer was also added, with further ice incubation for another 15 min. Centrifugation was done at 13000 g at 4°C for 15 min, supernatant and pellet collected in different tubes, 1.5 mL TE buffer added to pellets, with further addition of trichloroacetic acid (TCA), separate the supernatant with centrifugation and add DPA reagent to it. Allow overnight incubation to take place and record the OD values at 600 nm. The percentage of fragmented DNA was calculated by the following formula:

%DNA Fragmentation= (OD of supernatant)/(OD of supernatant+OD of pellet)

Statistical analysis

The data is illustrated depicting mean value ± standard deviation. Further comparison was done between groups and was analyzed with one-way analysis of variance (ANOVA) under Tukey's test using Prism software (Prism software Inc. CA). Significance level was accepted at $P \leq 0.05$ level.

Results

Nanoparticles size and surface zeta potential

Nanoparticles mean hydrodynamic diameter was evaluated using DLS technique and was shown to be 79.75nm, polydispersity index (PDI) of 0.281 was recorded for iron nanoparticles synthesized by polyol method but iron nanoparticles synthesized with co-precipitation method has size of about 135.1 nm, PDI of 0.35, as illustrated in (Fig. 1A & B). The lower PDI specify homogeneity of nanoparticles that are synthesized, apart from this it also indicates the narrowness of distribution spectra for synthesized nanoparticles and thus it confirms the uniform size distribution that can easily be further evaluated from SEM technique. Zeta potential was also determined as shown in (Fig. 1C & D) it was obtained to be 30 mV for nanoparticles synthesized by co-precipitation method, and for nanoparticles synthesized by polyol method it was found to be 25.2 mV and signify stability of the nanoparticles.

Antibacterial properties

Antibacterial activity evaluatedas ZOI

Antibacterial activity of iron nanoparticles was determined against *E. coli* and *S. aureus* based on Kirby-Bauer method the results are represented in graphical form (Graph 1), with the subsequent images represented in (Fig. 2). At different nanoparticles concentration such as 1 mg/mL, the ZOI values are 0.33±0.28 mm and 0.51±0.02 mm, for 10 mg/mL it is 0.66±0.28 mm and 0.51±0.02 mm in polyol synthesized iron nanoparticles but for co-precipitation method at concentration 1 mg/mL, the ZOI values are 0.33±0.28 mm and 1.16±0.28 mm, for 10 mg/mL it is 2.00±0.50 mm and 2.34±0.29 mm, for positive control ZOI values are 8.7±0.34 mm and 6.83 ±0.28 mm, no ZOI was recorded for negative control in *E .coli* and *S. aureus* bacterial species, respectively, suggesting nearly higher susceptibility of gram-positive bacteria than gram-negative bacteria for the synthesized nanoparticles[10].

Growth kinetics studies

This study was performed to investigate the inhibitory outcome of iron nanoparticles on turbidity of bacterial

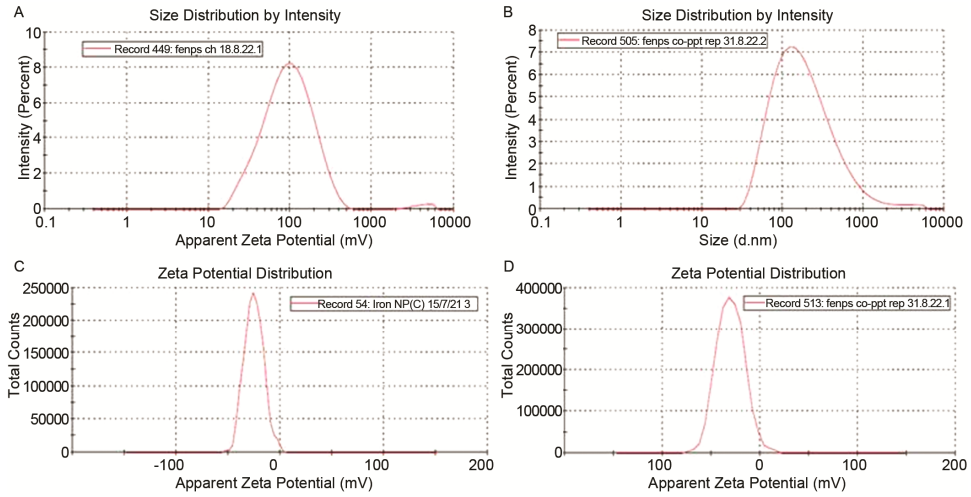
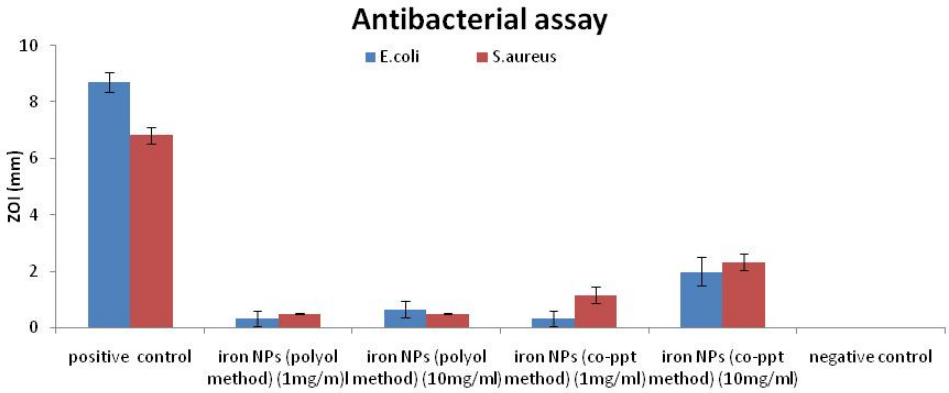


Fig. 1 — (A & B) Size distribution obtained from DLS with polyol method nanosize is of 79.75 nm and for co-precipitation method it is 135.1 nm; and (C & D) Surface zeta potential of the iron nanoparticles with polyol method is at –25.2 mV and for co-precipitation method it is at –30.4 mV



Graph 1 — Representation of data as ZOI values depicting the antibacterial effect of the synthesized iron NPs at different concentrations

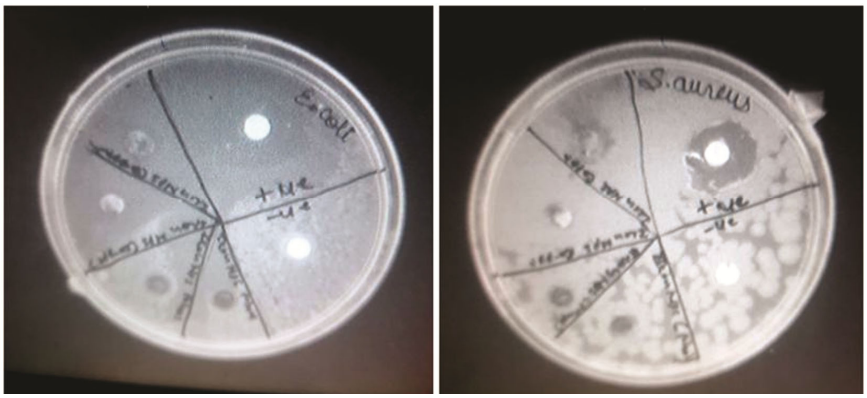


Fig. 2 — Comparative analysis of antibacterial activity of iron NPs against Gram positive *S. aureus* and Gram negative *E. coli* bacteria with gentamycin (5 mg/mL) as a positive control and sterilized discs as negative control

culture. Figure 3 noticeably shows a variation in growth on comparison to that of normal culture. Treatment with iron nanoparticles resulted in inhibition of 24% and 20% in the growth of *S. aureus* and *E. coli* post 24 h of incubation with polyol method synthesised iron nanoparticles. Similarly, for co-precipitation based iron nanoparticles showed higher growth inhibition of 33% and 41%. This can be attributed of effect introduced by nanoparticles causing bacterial damage.

DNA assay

Percentage of DNA fragmentation in *S. aureus* and *E. coli* bacterial genomes represented in (Fig. 4) for iron nanoparticles synthesized differently against negative control. This DPA reaction is a quantitative estimation that takes advantage of the bonds between purines and deoxyribose, which are very labile. Once these bonds are broken, inorganic phosphates are liberated from the DNA and provide the substrate w-hydroxylevulinylaldehyde which under acidic conditions reacts with DPA & forms a blue-colored complex. The estimated amount of fragmented DNA is slightly higher in gram-negative bacteria as compared to gram-positive bacteria. The percentage of fragmented DNAs in a control group is 33% ± 4.94% and 28% ± 1.65% in *E. coli* and *S. aureus* but for iron nanoparticles from the polyol method and co-ppt method showed the fragmented DNAs of about 50% ± 1.70% and 43% ± 0.12%; 55%± 0.39% and 51%± 1.30% in *E. coli* and *S. aureus*, respectively. When it comes to the discussion around the antibacterial properties, the mechanism of action is not well understood yet it can be elucidated as an bactericidal/static effect introduced in a form of electrostatic/ direct interaction between bacterial species and the synthesized nanoparticles leading to nanoparticles internalization within the cells causing cell membrane disruption, increased production of reactive oxygen species, causing DNA damage in form of fragmentation in bacterial genomic DNA, *etc* all represented in (Fig. 5). A lot of studies have signified the presence of contradictory reports representing *E. coli* having elevated antibacterial effect with increasing

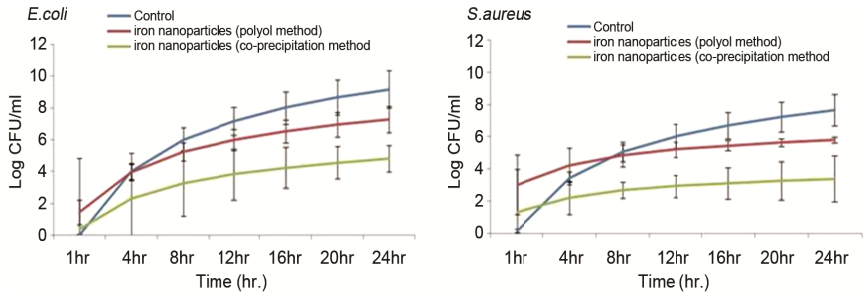


Fig. 3 — Growth kinetics curve (A) Gram negative bacteria *E. coli*; and (B) Gram positive bacteria *S. aureus*, when treated with iron NPs synthesised with two different method. Log CFUs plotted as a function of time, data represented as mean ± S.D

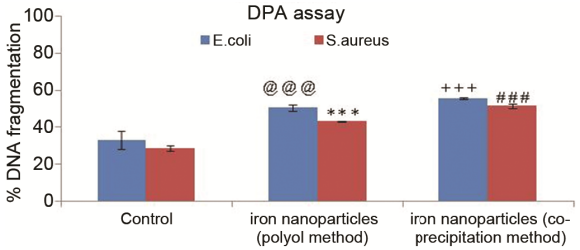


Fig. 4 — Percentage DNA fragmentation and comparison of iron nanoparticles prepared from different methods against negative control

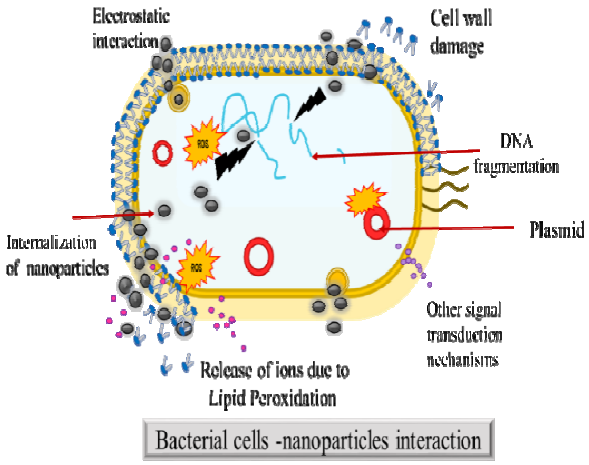


Fig. 5 — Schematic representation of bacterial cells-nanoparticles interaction: Iron nanoparticles in the extracellular space can (1) interact with either be confined to the extracellular environment, or enter the cells *via* endocytosis (2) or passive diffusion (3). Once internalized, both Iron nanoparticles and the ions dispense in the cytoplasm. Both ions and nanoparticles induce oxidative stress by increasing ROS production, leading, lipid and protein peroxidation, with cell membrane damage, and the genomic DNA damage

concentration, while some represents no activity against pathogen. It can thus be inferred that alone these nanoparticles are quite effective. Modification in the synthesized nanoparticles substantially can improve the antibacterial potential hence it can potentiate its utilization as an antibacterial formulation against bacterial growth.

Data represented as mean values ± Standard deviation (n=3)

@denote significant variation of control *vs* iron nanoparticles (polyol method) treated group in *E. coli*

*denote significant variation of control *vs* iron nanoparticles (polyol method) treated group in *S. aureus*

+denote significant variation of control *vs* iron nanoparticles (co-precipitation method) treated group in *E. coli*

#denote significant variation of control *vs* iron nanoparticles (co-precipitation method) treated group in *S. aureus*

*P <0.05; ** P <0.01; *** P <0.001

Conclusion

The application of nanotechnology and respective NPs is vital for many biological and Medicare sectors as it has relevance due to the occurrence of physiological activities on nano scale range. Harmonizing the off-putting influence at the cell level and exploiting the probability of NPs for formulating effective nano medicine. Hence, determining its effectiveness based on the approach of action and significant impact of coatings material on micro-biological activity. The multitarget approach can also be achieved with the application of such NPs being effective against microbial growth. Our work exhibit the successful synthesis of NPs and its application in antimicrobial avenues. NP-based research has the prospective of generic treatment strategies for enhanced effectiveness as antibacterial agents.

Conflict of interest

All authors declare no conflict of interest.

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