Brief Report

Antimicrobial Effect of Imipenem-Functionalized Fe_2O_3 Nanoparticles on *Pseudomonas aeruginosa* Producing Metallo β-lactamases

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Background: Resistant strains of *Pseudomonas aeruginosa* to imipenem was medical treatment problem, especially in burnt units of hospitals.
Objectives: This study was conducted to evaluate the antimicrobial effect of Fe_2O_3 nanoparticles alone and functionalized with imipenem on *P. aeruginosa* starins producing metallo β-lactamases (MBL).
Materials and Methods: A disk diffusion method was used to isolate a clinical *P. aeruginosa* producing Metallo β-lactamases with imipenem resistance. The minimum inhibitory concentration (MIC) of Fe_2O_3 nanoparticles and imipenem were calculated against the bacteria. The antimicrobial effect of nanoparticles functionalized with the antibiotic was determined. Standard strain of *P. aeruginosa* ATCC: 27853 was used as control.
Results: The clinical sample was resistant to imipenem (up to 28 $\mu g.mL^{-1}$). Similarly, MIC of the nanoparticles against the isolate was 160 $\mu g.mL^{-1}$. Subsequently, the combination of 16 $pg.mL^{-1}$ of antibiotic with 80 $\mu g.mL^{-1}$ of Fe_2O_3 nanoparticles were able to inhibit the growth of the isolate.
Conclusions: Fe_2O_3 nanoparticles functionalized with imipenem can impair antibiotic resistance mechanisms of bacteria as it can make the imipenem resistant the aforementioned bacterium more susceptible to weaker concentrations of antibiotic. It also has its own antibacterial effect in certain concentrations.

Keywords: Antimicrobial properties; Fe_2O_3 nanoparticles; *Pseudomonas aeruginosa*

1. Background

Pseudomonas aeruginosa is amongst one of the most important pathogens in burnt units and one of the main causes of nosocomial infections (1). These bacteria are rampant within the environment and they are easily isolated from hospital settings and medical personnel (2). Due to the widespread use of antibiotics in recent years, the noted strain has developed a resistance towards broad spectrum of antibiotics (2). Consequently, the existence of metallo β-lactamases (MBL) *in P. aeroginosa* which considers as a major issue in treatment options (3). Imipenems have widely been used as the mainstay for the treatment of severe infections caused by *P. aeruginosa,* because they can easily permeate through the porins on the outer membrane of these bacteria. Additionally, they have a structure resistant to hydrolytic activities of most β-lactamases and have a high affinity for penicillin-binding proteins (PBP).

Imipenem-resistant of *P. aeruginosa* has emerged and is spreading worldwide (4). Previous studies showed that MBL strains of *P. aeroginosa* are one of the main concerns in Tehran hospitals. This is evident as 97.5% of the infectious strains in Motahari Burnt Center in Tehran have been identified as *P. aeruginosa*, which are treated by imipenem as the last line of treatment (5, 6). Resistance to imipenem is increasing as these strains can successfully acquire resistance genes (7). Consequently, finding innovative ways to overcome this drug resistance is considered vital. Evidently, the previous studies illustrated that the use of silver nanoparticles combined with the antibiotic

streptomycin can reduce the streptomycin minimum bactericidal concentration (MBC) against *Brucella abortus* increasing the anti-Brucella activity (8).

Many studies showed the antibacterial activity of metal oxide nanoparticles (9). It is thought that this action can reduce the resistance mechanisms and increase the sensitivity of these bacteria to low concentrations of antibiotics. The effect of Fe_2O_3 nanoparticles functionalized with imipenem antibiotic was examined on MBL *P. aeruginosa*. A predefined concentration of nanoparticle was enhanced the effects of imipenem against the resistant bacteria. By this mean a new way for treatment of antibiotic resistance strains can be introduced. Last but not least and in line with implementing physical and chemical parameters in functionalizing nanomaterials, new frontiers in controlling the bacterial pathogens can be introduced.

2. Materials and Methods

2.1. Bacterial Isolates

This fundamental- applied study was conducted on the *P. aeruginosa*, isolated from clinical specimens taken form burnt hospitalized patients in Motahari Hospital in Tehran. Furthermore the noted bacterial isolates were submitted to the Molecular Biology Research Center of Baqiatallah University of Medical Sciences and cultured on Muller-Hinton agar medium (Merck, Germany for 24 h at 37ºC. For quality control test, *P. aeruginosa* ATCC: 27853 was provided from Pasteur Institute of Iran.

2.2. Antibiotic Sensitivity of the P. aeruginosa Strain

Antimicrobial susceptibility tests were performed based on Kirby-Bauer disk diffusion method. Suspension culture of positive clinical sample from *P. aeruginosa* was prepared as 0.5 McFarland, and cultured on Mueller Hinton agar medium (Merck, Germany). The antibiotic disks contained imipenem (10 pg), gentamicin (10 pg), ceftazidime (30 pg) and penicillin (10 units), and penicillin, and incubated at 37ºC for 16h. Inhibition zone was measured and a clinical sample resistant to all antibiotics was chosen according to the evaluated Clinical and Laboratory Standards Institute (CLSI) guideline. The standard strain of *P. aeruginosa* ATCC: 27853 was used as control.

P. aeroginosa was prepared at a concentration equivalent to 0.5 McFarland and 100 mL of the prepared solution was added to the microplate wells containing 100 ml of 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5 $\mu g.mL^{-1}$ of pure imipenem antibiotic and 100 mL Mueller Hinton agar medium. The microplate was incubated for 24 h at 37°C. The *P. aeruginosa* ATCC: 27853 standard strain was used as control.

2.3. PCR Assays

To confirm the presence of imipenem resistance genes, primer pairs, 5′-CGG CC G/T, CAG GAG A/CGG /T, CTT, T-3′ and 5′-AAC CAG TTT TGC C/T TT AC C/T AT-3′ were designed. The primers amplified a fragment of *blaIMP* (Figure 1). *P. aeruginosa* 68549 that containing the resistance gene was used as a positive control.

2.4. Determination of Minimum Inhibitory Concentration for Fe_2O_3 Nanoparticles

Fe_2O_3 nanoparticles (99%, 50-20 nm) were purchased from Pishgaman Nano-mavvad Company (Tehran, Iran). Based on Clinical Laboratory Standards institute (CLSI) recommendations, the minimal inhibitory concentration (MIC) were determined for the sample exposed to nanoparticle suspensions. MIC defined as the lowest concentration of nanoparticles, which can inhibit the growth of organisms in culture medium. Tubes containing culture medium as a batch system with different concentrations of Fe_2O_3 nanoparticles suspension, were prepared With concentration of, 200, 180, 160, 140, 120, 100 $pg.mL^{-1}$. The tubes containing nanoparticles and bacteria were incubated for approximately 24 h in a shaking incubator

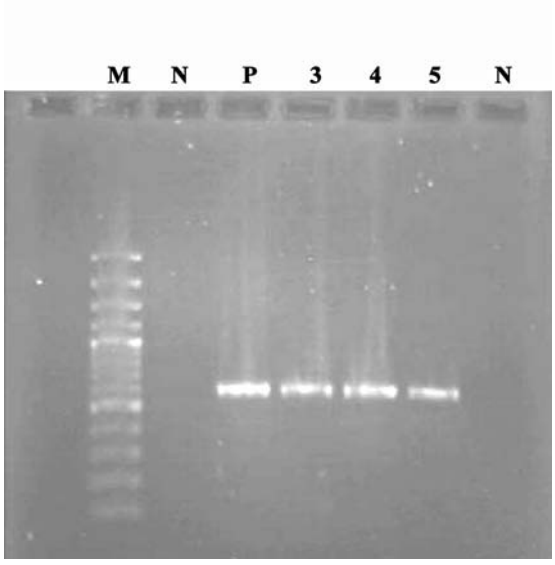

Figure 1. PCR detection of blaIMP resistance gene. M: Gene Ruler 100 bp DNA Ladder (Thermo Fisher Scientific-Catalog number: SM0241), N: negative control, P: Standard *P. aeroginosa* 68549 strain containing *blaIMP* (587 bp). Lane 3, 4 and 5: Amplicon of an isolate resistant to imipenem (587 bp)

(120 rpm, temperature of 37°C). All the above steps were repeated for 3 times to reduce experimental errors.

2.5. Functionalizing Fe_2O_3 Nanoparticles with Antibiotics

The concentration of antibiotic (16 mg.mL^{-1}) with no effect on bacteria was selected and added to Fe_2O_3 nanoparticles with concentrations of 140, 120, 100, 80, 60, 40 µg.mL^{-1}. The final mixtures were evaluated according to CLSI standards for calculating MIC.

The values obtained were used for functionalizing Fe_2O_3 nanoparticles with 80 µg.mL^{-1} in the presence of 16 µg.mL^{-1} imipenem. Functionalizing was performed by mixing 1 µL of each nanoparticle and antibiotic followed by sonication (3 cycles for 30 seconds). The mixture let to settle for 24 h, allowing thioether (sulfides) group transfer from imipenem to the surface of nanoparticles that is called nanoparticle functionalizing. Unbounded antibiotic molecules were removed by 5× centrifugation at 15000 ×g and 12ºC. After each step, the supernatant was removed and the remnant pellet was washed with sterile distilled water. Eventually the solution was kept at dark in 4°C.

3. Results

3.1. Results of Antibiotic Sensitivity

The selected clinical isolate was identified through biochemical tests and resistant to all antibiotics including imipenem (10 pg.mL^{-1}), gentamicin (10 pg.mL^{-1}), ceftazidime (30 pg.mL^{-1}) and penicillin (10 units). Similarly, it was resistant to increasing concentrations of imipenem. Because of the producing MBL enzymes, the isolate was considered as a super multidrug resistance the aforementioned bacterium.

3.2. PCR Test Results

The designed primers were tested for specificity and sensitivity of using DNA templates prepared from From control strains known as producing β-lactamases. The primers showed 100% specificity and 100% sensitivity for detecting MBL enzymes. This test was able to identify P. aeroginosa producing MBL enzymes (blaIMP resistance gene) Based on uniplex PCR.

3.3. The Results of Fe_2O_3 Nanoparticles Synthesis

The typical XRD patterns Fe_2O_3 nanoparticles are shown in Figure 2. The results of Fe_2O_3 nanoparticles by XRD also revealed their polycrystalline structure. The effect of their shape and size nanoparticles on the antimicrobial properties that were investigated by TEM electron micrograph (Figure 2).

3.4. The Result of Antimicrobial Effect of Nanoparticles Functionalized

The antimicrobial effect of silver nanoparticles is presented in Figure 3. According to the positive and negative ODs, the MIC of nanoparticles was 160 µg.mL^{-1}. Thus, in this report, Fe_2O_3 nanoparticles have shown the antibacterial behavior.

3.5. The Result of Antimicrobial Effects of Nanoparticles Functionalized

The antimicrobial effect of silver nanoparticles

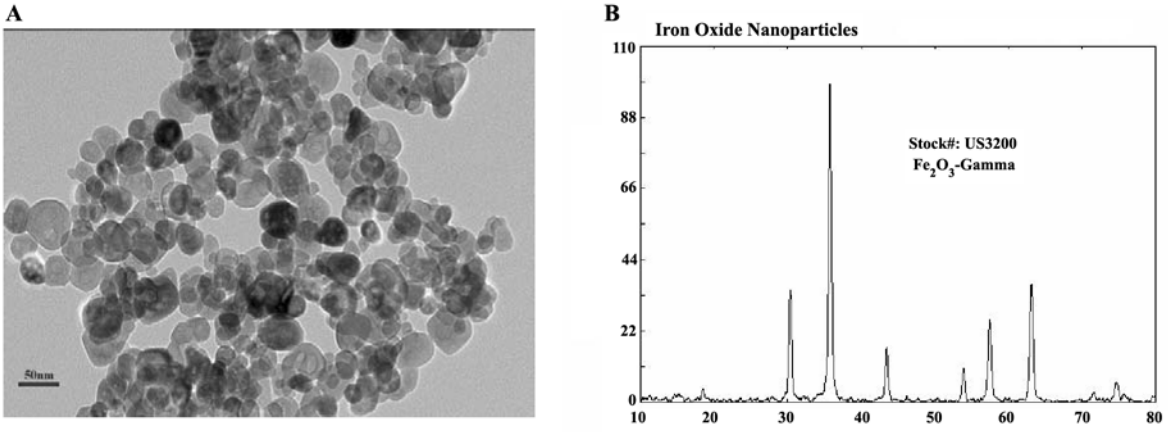

Figure 2. A: TEM electron micrograph of Fe_2O_3 nanoparticles, B: X-ray diffraction pattern (XRD) of Fe_2O_3 nanoparticles shows the structure. Initial peaks are indicating polycrystalline structure. Similarly, the secondary peaks are indicating the form of nanocomposites

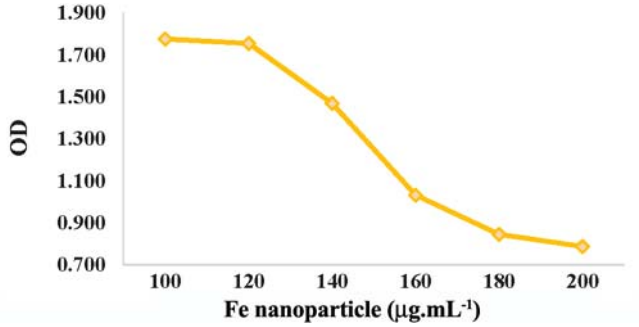

Figure 3. The antimicrobial result of Fe_2O_3 nanoparticles against *P. aeroginosa* isolate studied with ELISA reader. The positive and negative control ODs were reported to be as 1.913 and 0.775, respectively

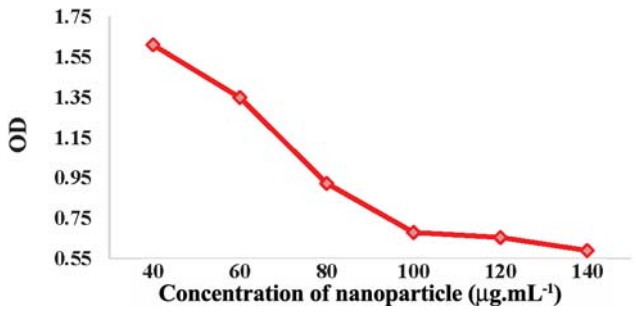

Figure 4. Results of antimicrobial effects of Fe_2O_3 nanoparticles against *P. aeruginosa* producing metallo β-lactamases, studied with ELISA reader. The negative and positive control ODs were 0.659 and 1.747, respectively

functionalized with imipenem is presented in Figure 4. Antibacterial activity results revealed that Fe_2O_3 nanoparticles functionalized with imipenem acted as excellent antibacterial agents against *P. aeruginosa* compared to Fe_2O_3 nanoparticles. According to The positive and negative ODs, the MIC of nanoparticles was 80 µg.mL^{-1}.

4. Discussion

The antimicrobial effect of Fe_2O_3 against the *P. aeuroginosa* producing metallo β-lactamases is to be studied. Here, the antimicrobial effect of Fe_2O_3 with inhibitory role in bacterial growth was analyzed. The result was clearly indicative of the resistance of *P. aeuroginosa* to increasing concentration of imipenem and other common antibiotics. This means controlling such bacterial isolates would be impossible via antibiotic treatment. Inactivity of porin cahnnels (OprD) in one hand and the activity of beta lactamases (AmpC) related to chromosome, in the others seems the causative agents of high resistance towards imipenem (10-12). Furthermore, the role of antibiotic efflux pumps align with the production of metallo-beta-lactamases can both contribute to the resistance towards carbapenems (13, 14).

In line with resistance mechanisms, it is hypothesized that application of low concentrations of Fe_2O_3 nanomaterials may negatively affect the functionality of porin pumps. Moreover, these particles may occupy the active site of metallo-beta-lactamases enzyme. This allows the transfer of low concentrations of antibiotics into the the aforementioned bacterium, increasing their antimicrobial property. The results suggest that in a competitive environment, nanoparticles with polycrystalline structure and 50-30 nm may be able to implement their antimicrobial properties more quickly than the antibiotics.

Antimicrobial nanoparticles deal many distinctive advantages in reducing overcoming resistance, acute toxicity and low cost, when compared to conventional antibiotics. So he study confirms Fe_2O_3 nanoparticle MIC as 160 mg.mL^{-1}; however, the analysis of the synergistic effect of nanoparticle and antibiotic confirms that the half concentration of the nanoparticle along with 16 µg.mL^{-1} of antibiotic can be an affective mixture minimizing the bacterial growth suggesting a new treatment option.

Azam *et al*. (2012) reported the antimicrobial property of metal nanoparticles including Fe_2O_3 using well plate method (8). The study confirmed that Fe_2O_3 nanoparticles growth inhibitory role on standard *P. aeroginosa* strain. The inhibition zone was reported to be 3 mm. Here, the growth of the resistant bacteria was successfully hampered even at lower concentrations of antibiotics and nanoparticles.

Fe_2O_3 nanoparticle is a notable new antimicrobial agent that can have synergistic effect with antibiotics in counteracting with bacterial resistance. This can potentially be served as a new treatment protocol subject to further evaluation.

Acknowledgements

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