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Antimicrobial Effects of Iron Oxide Nanoparticles in the Presence of Dispersing Agent

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The iron oxide (IO) nanoparticles have many potential applications in biology due its magnetic properties. In this study, we analyzed antimicrobial activity of two important (IO) nanoparticles (γ-Fe_2O_3, Fe_3O_4). These (IO) nanoparticles were characterized by dynamic light scattering (DLS) and transmission electron microscopy (TEM) and the starch were used as dispersing agent in order to reinforce the colloidal stability of IO nanoparticles. The DLS results showed the average size of IO nanoparticles were an about 30-40 nm and TEM results demonstrated that IO nanoparticles were well dispersed with starch. Further, *Staphylococcus aureus* (PTCC 1431), *Escherichia coli* (PTCC 1395), *Pseudomonas aeruginosa* (PTCC 1599) and *Candida albicans* (PTCC 5027) were grown in the presence of five different IO nanoparticles concentrations with and without starch. 3-[4,5-dimethylthiazol- 2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction and agar well diffusion assays were performed and the results provide evidence that γ-Fe_2O_3 and γ-Fe_2O_3/starch nanoparticles did not inhibit microbial growth even at the highest concentration (50 mg/mL). But Fe_3O_4 nanoparticles inhibited microorganisms growth, specially at the highest concentration (50 mg/mL) and at the present of starch. As a result, starch as dispersing agent can increase the antimicrobial effects of Fe_3O_4 nanoparticles.

Key words: Iron oxide nanoparticles (γ-Fe_2O_3, Fe_3O_4), Antimicrobial effects, dispersing agent, starch.

In recent 20 years, a rapid increase in the emergence of antibiotic-resistant bacteria that leading to elevated bacterial pathogenesis at the global level has been observed[1-3]. *S. aureus* is one of the most important antibiotic resistant bacteria that possess an increasing ability to resist antibiotics (such as penicillin, tetracycline, erythromycin, and vancomycin)[4-6]. This bacterium is responsible for prosthetic infection (such as through the use of catheters, endotracheal tubes, and other biomaterials) in addition to local infections[7-9]. *E.coli* is the most common bacterium that causes urinary tract infections, or UTIs [10], this bacterium acquired the ability to resistance against these antibiotics (Ampicillin, Streptomycin, Sulfa drugs, and Quinolone drugs)[11].

P. aeruginosa is an opportunistic pathogen that leading cause of nosocomial infection particularly in patients with severe burns and in cancer and AIDS patients who are immunosuppressed and it is intrinsically resistant to multiple classes of antimicrobial agents[11-14]. Antifungal drug therapy is not exception; fungi are eukaryotic organisms with a similar structure and metabolism to their eukaryotic hosts. Also they gained the ability to resist many types of antifungal drugs. Therefore, finding alternative treatment for various infections from bacteria and fungi that they are unable to acquire resistance and with lower side effects is necessary[15].

Iron oxide nanoparticles because of its magnetic properties have gained wide attention in

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biology[16]. These nanoparticles with sizes less than 15nm, are super-paramagnetic at room temperature[17] and under 100 nm, have been developed as magnetic resonance imaging (MRI) contrast agent[18, 19] cell separation, as hyperthermia agents[20, 21] and a drug delivery system can be developed by using an external magnetic field to target site in bacterial infections (such as implant infection)[22].

Although the mechanism of iron oxide nanoparticle could kill bacteria is not fully understood, but there are some hypotheses that attributed to the generation of reactive oxidative species (ROS). Metal oxide nanoparticles can generate ROS in a Fenton-type reaction, in which hydrogen peroxide is reduced by ferrous ions to form extremely active hydroxyl-free radicals and lead to microorganisms damage[23, 24]. However, because of the hydrophobic interactions between the particles, they agglomerate and form large clusters, resulting in an increased particle size and a low colloidal stability. The clusters exhibit strong magnetic dipole–dipole attractions and show ferromagnetic behavior[25]. Therefore in this study starch were used as dispersing agent, which is formed of repeating 1, 4—D glucopyranosyl units: amylose and amylopectin[26].

Developing fast and accurate antimicrobial susceptibility assay is important for determine the viability rate of microorganisms. So in this article MTT assay were used instead of traditional methods such as Minimum Inhibitory Concentration (MIC) and enumeration of colony-forming units (CFU). MTT is a yellow tetrazolium salt that reducing into water insoluble purple formazan crystals by cellular dehydrogenases. So when the microorganisms are viable the increase in concentration of dissolved formazan in an organic solvent is visible and the lack of integrity of cell membranes can greatly reduce the cellular MTT reduction activity[27-30].

MATERIALS AND METHODS

Characterization of IO nanoparticles

To measure the hydrodynamic size of the nanoparticles, 100 µL of the particle solution was diluted with 1.5 mL of water and placed into a cuvette of a Zetasizer-nano instrument (ZS3600, England). Experiments were conducted in triplicate to obtain an average number-size distribution.

A droplet of IO nanoparticles was placed on a TEM copper grid and allowed to dry. The imaging was carried out at 100 kV on a Philips EM208 TEM (Philips, Holand) and size calculations were carried out with Image.

Dispersing with Starch

0.1 gr of starch were dissolved in hot deionized water to produce Starch solutions. Then the different concentrations of IO nanoparticles were poured into the prepared starch solution under vigorous stirring at 60°C for 2 h.

Microbial Analysis

Microbial culture

Staphylococcus aureus with Persian Type Culture Collection (PTCC 1431), *Escherichia Coli* (PTCC 1322), *Pseudomonas aeruginosa* (PTCC 1599) and *Candida albicans* (PTCC 5027) were obtained in frozen form. A single colony of microorganisms was selected and inoculated into centrifuge tubes containing 5 mL of Muller-Hinton Broth for bacteria and 5ml of Sabrv dextrose broth for *Candida albicans* until the turbidity of them reach to 0.5 McFarland standard tubes (1.5×10^8 cells mL^{-1}).

MTT assay

180 µl of PBS were seeded in a 96-well plate and 20 µl of microorganism suspension (0.5 Mc farland) were inoculated and cultivated for 24 h. After that the microorganism were treated with 100 µl of γ-Fe_2O_3 and Fe_3O_4 (concentration ranged from 2.5 to 50 mg/ml) with and without starch for 48 hour. The untreated microorganisms suspension served as a positive control. At the end of the treatment period, MTT (final concentration 5 mg/mL in PBS) (Sigma, St. Louis, USA) was added to each well, which was then incubated at 37 °C in 5% CO_2 for 2 h.

Then DMSO (dimethyl sulfoxide) (Sigma, St. Louis, US A) were used to dissolved formazan colored crystals. The absorbance was measured at 550 nm via microplate reader. A change in colour from yellow to violet indicated growth of microorganisms.

Viability percent of bacteria was calculated as a ratio of the absorbance of the treated group divided by the absorbance of the positive control group, multiplied by 100 to give percentage of the proliferation.

Agar well diffusion assay

20 µl of each bacterial suspension (0.5 Mc farland) and 20 µl of *candida albicans* suspension (0.5 Mc farland) were spread out on the surface of Muller-Hinton agar and Sabrv dextroseagar by sterile swap respectively. Then the different concentrations of IO nanoparticles with and without dispersing agent were poured in to the wells. After 48h of the incubation at 37°C the inhibition zone were investigated.

RESULTS AND DISCUSSION

IO nanoparticles characterization

The hydrodynamic diameter measurement results showed that, the IO chain-like particles had an average size of 30-40 nm.

The agglomerated clusters of unused dispersing agents of γ-Fe_2O_3 nanoparticles can clearly be seen in Fig. 2.A. Starch with negative charge could chemisorbed on the surface of the nanoparticles with positive charge, and at the other hands IO nanoparticle has hydrophobic interactions but starch has strong hydrophilic and biodegradable behaviors which makes the particles hydrophilic, thus these nanoparticles become dispersible in deionized water (Fig. 2.B).

Also the difference between the agglomerated clusters of Fe_3O_4 nanoparticles and dispersible form of them can clearly be seen in Fig. 3.A. and figure 3.B respectively.

Antimicrobial activity of Io nanoparticles

After 48 hours of incubation, Fe_3O_4 nanoparticles was able to show a concentration dependent antimicrobial behavior. The both results of MTT assay and agar well diffusion assay demonstrated that there was no significant difference in each four microorganisms' numbers at the presence of low concentration of Fe_3O_4 nanoparticles (2.5 mg/ml) and when the concentration was reached to 5 mg/ml an increase in bactericidal effect of Fe_3O_4 nanoparticles to *S. aureus* and *E. coli* was observed. But this bactericidal effect to *P. aeruginosa* and *C. albicans* was observed just when the concentration increased up to 7.5 mg/mL (Fig. 4, 5).

The MTT results of γ-Fe_2O_3 nanoparticles after finished the same time as Fe_3O_4 nanoparticles, didn't show any different with control sample even at highest concentration (50 mg/mL) of γ-Fe_2O_3 nanoparticles. Also the similar result was observed with the agar well diffusion assay (Fig. 5).

Principally, the concentration of nanoparticles is a major contribution to microorganisms activity inhibition. Taylor et al. observe a similar concentration-dependent behavior when they investigated the antimicrobial

Table 1. The agar well diffusion assay results for different concentrations of Fe_3O_4 nanoparticles on test microorganisms

Samples	2. 5 mg/ml	5 mg/ml	7. 5 mg/ml	10 mg/ml	50 mg/ml
S. aureus	-	8 mm	14mm	17mm	28mm
E. coli	-	5 mm	10mm	13mm	21mm
P. aeruginosa	-	-	5mm	9mm	16mm
C. albicans	-	-	5mm	7mm	13mm

Table 2. The agar well diffusion assay results of dispersed Fe_3O_4 nanoparticles on test microorganisms

Samples	2. 5 mg/ml	5 mg/ml	7. 5 mg/ml	10 mg/ml	50 mg/ml
S. aureus	-	9 mm	15mm	28mm	38mm
E. coli	-	5 mm	12mm	25mm	36mm
P. aeruginosa	-	-	5mm	9mm	26mm
C. albicans	-	-	7mm	9mm	26mm

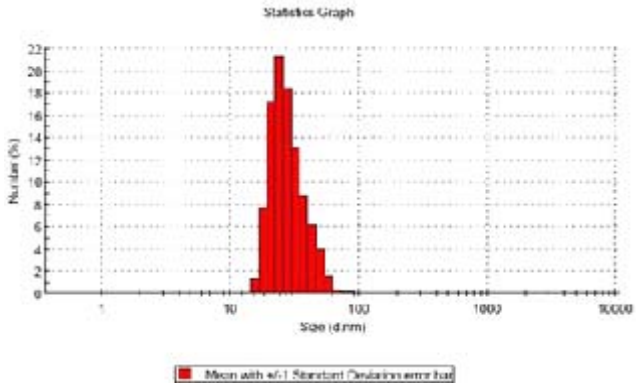
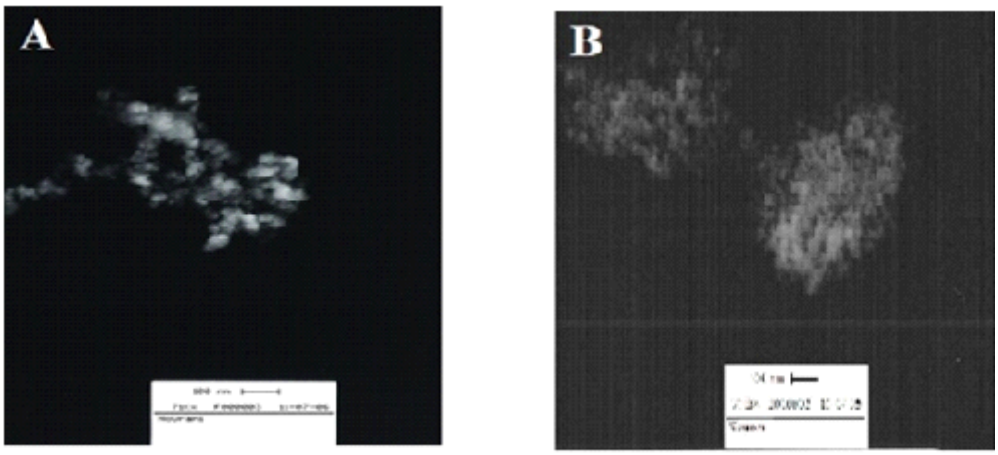


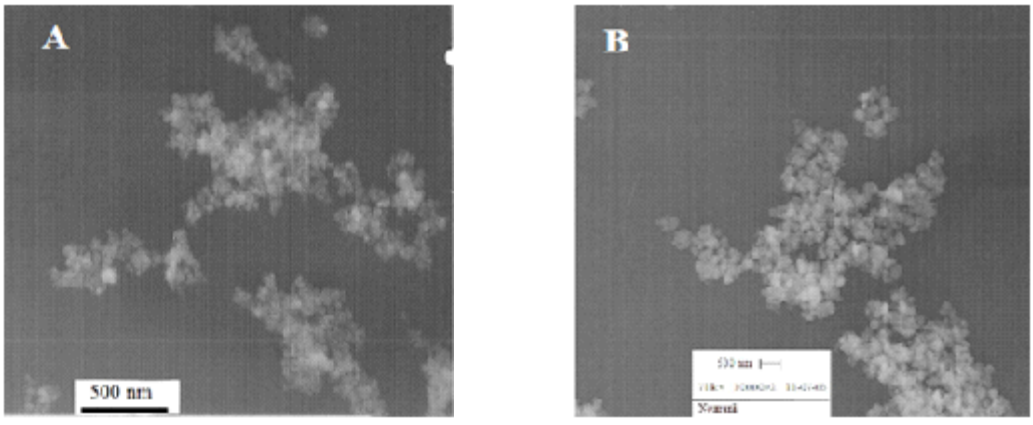
Fig. 1. IO nanoparticle size distribution as measured by dynamic light scattering



a) Agglomerated clusters of γ-Fe_2O_3 nanoparticles

b) Dispersed γ-Fe_2O_3 nanoparticles.

Fig. 2. TEM images of γ-Fe_2O_3 nanoparticles



a) Agglomerated clusters of Fe_3O_4 nanoparticles

b) Dispersed Fe_3O_4 nanoparticles.

Fig. 3. TEM images of the Fe_3O_4 nanoparticles

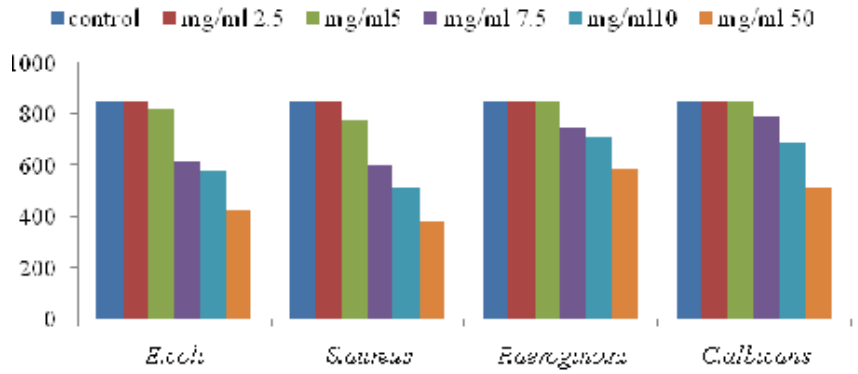
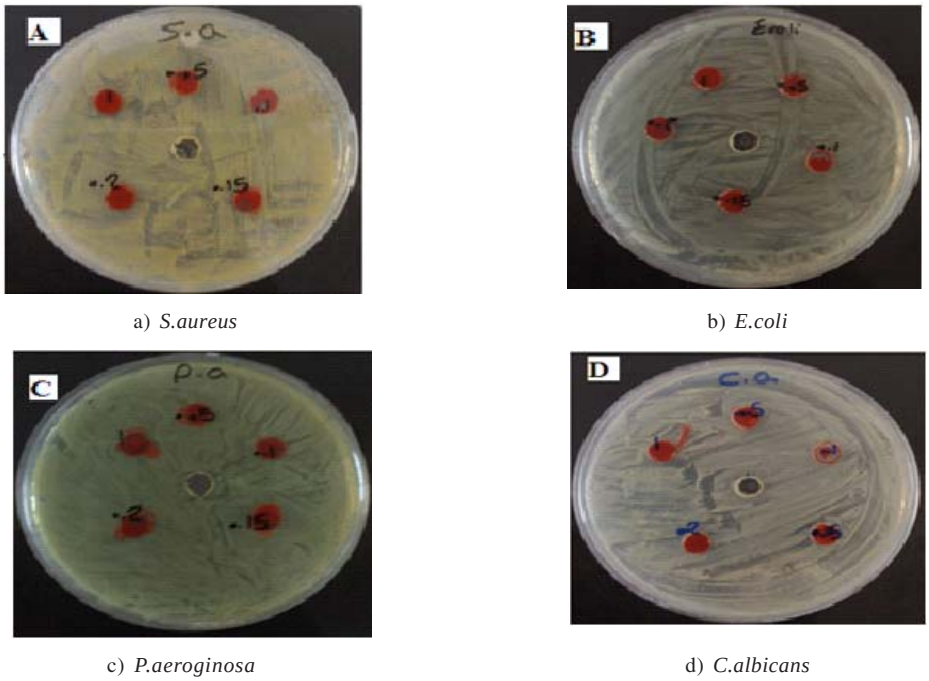


Fig. 4. The results of Fe_3O_4 nanoparticles MTT assay on test microorganisms



a) *S.aureus* b) *E.coli*

c) *P.aeroginosa* d) *C.albicans*

Fig. 5. The agar well diffusion assay results of different concentrations of γ-Fe_2O_3 nanoparticles

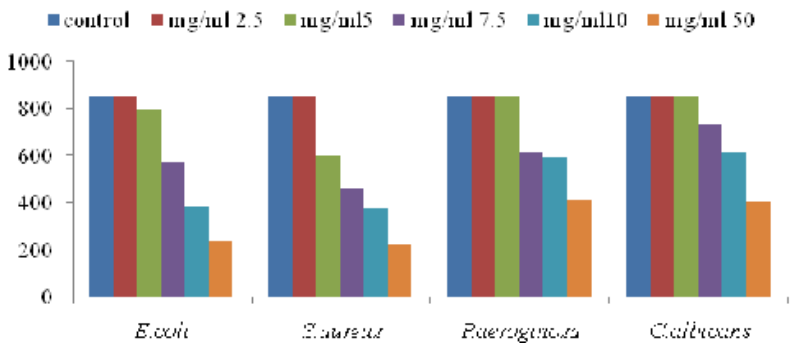


Fig. 6. The results of dispersed Fe_3O_4 nanoparticles MTT assay on test microorganisms

effects of IO nanoparticles (100 µg/mL, 1 mg/mL, and 2 mg/mL) on *S. epidermidis*[31]. Tran *et al.*, also reported IO/PVA nanoparticles inhibited *S. aureus* growth at the highest concentration (3 mg/mL) when *S. aureus* was grown at the presence of three different IO nanoparticle concentrations (30 µg/mL,300 µg/mL, and 3 mg/mL)[32].

There are several factors that caused IO nanoparticles to have antimicrobial properties moreover the concentrations of them. In present study the relationship between the dispersing of nanoparticles and antimicrobial effects of them was observed by Fe_3O_4 nanoparticles. The chemisorptions of starch on the surface of IO nanoparticles presents strong hydrophobic behaviors, thus these dispersible nanoparticles can produce more oxidative stress that cause killing of microorganisms (Fig. 4,6,7).

But γ-Fe_2O_3 nanoparticles didn't show any different at the present of starch, it is may be because of its redox state. Actually the generated ROS from these nanoparticles was not enough to kill microorganisms. IO nanoparticles produce ROS, including superoxide radicals (O_2^-), hydroxyl radicals (-OH), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) that generated oxidative stress leading damage to proteins and DNA in bacteria[24]. In this case keenan *et al.,* reported, when Fe^{2+} reacts with oxygen can produce H_2O_2 [32]. This H_2O_2 consequently react with ferrous irons via the Fenton reaction and produce hydroxyl radicals which are known to damage biological macromolecules[34]. The existence of relationship between the redox states of iron based nanoparticles and their cytotoxicity toward a Gram-negative bacterium, *E. coli* was investigated with Auffan *et al.,* They understood the cytotoxic effects of iron oxide nanoparticles appear to be associated with an oxidative stress as when they using a mutant strain of *E. coli* that completely devoid of superoxide dismutase activity[35].

CONCLUSIONS

The dispersing of IO nanoparticles with starch was carried out successfully and it was characterized with TEM, Zeta size, MTT and agar well diffusion tests for antimicrobial assays showed that the IO nanoparticles which dispersed with biocompatible starch can increase antimicrobial effect than undispersed IO nanoparticles. This dispersing agent with antimicrobial and magnetic properties can be used for medical applications such as magnetic hyperthermia method.

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