The Study of Effect of Superparamagnetic Iron Oxide Nanoparticles (SPION or Fe_3O_4 Nanoparticles) on *Candida albicans* Biofilm Formation

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ABSTRACT

Background and Objectives: *Candida albicans* is one of the most common fungal pathogens in biofilms that is widely seen in medical surfaces and medical devices, and in recent years has shown increased resistance to antifungal agents. In this experimental study, we aimed to study the Superparamagnetic Iron Oxide Nanoparticles (Fe_3O_4 nanoparticles or SPION), as a nanotechnology, on biofilm formation of *C. albicans*.

Methods: In order to evaluate the effects of SPION on *C .albicans*, the SPION were synthesized by chemical co-precipitation method. The formation of nanoparticles confirmed by FTIR and X-ray diffraction. Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of SPION were determined. Then effects of these nanoparticles on biofilms formation of *C. albicans* yeast were investigated by using of ELISA micro-titer plate method. Data were analysed by using variance analysis, Minitab and SPSS soft-wares ($P < 0.05$).

Results: The X-ray diffraction indicated that the SPION have a mean diameter about 70 nm. MIC and MFC of SPION on *C. albicans* were recorded 100 and 200 ppm in which biofilm formation reduced 87.2 and 100 percent, respectively. Nanoparticles had significant effect on reduction and inhibition of growth & biofilm formation by *C. albicans* yeast.

Conclusion: In light of the findings of this study, it seems that iron metal oxide nanoparticles may be a good candidate of a novel family of fungicidal compounds, but for human usage, it needs further research.

Keywords: *Candida albicans*, Biofilms, SPION, Nanoparticles

INTRODUCTION

Candida species are a kind of yeast from the *Blastomycetes* class of the *Cryptococcaceae* family (1). *Candida* species are of the natural microbiota, an individual's mucosal oral fissure, gastrointestinal tract and vagina (2), responsible for the different clinical advent from the mucocutaneous excessive growth of blood- stream infections (3). *Candida albicans* is distinguished by multiple virulence agents such as stick, secretion of proteases and production of biofilm (4).

The complementary stages of biofilm are multi-stage, which involves the intimacy of microorganisms, the maintenance of cellular interactions and communication with other cells, the formation of colonies, persistence to the surface and quorum sensing (5). Commonly, biofilms are single or multiple species of microbial communities stuck to a biotic and abiotic surfaces and packed in an extracellular polymeric substance (EPS) (6). Biofilm has appeared as a considerable cause of clinical problem from the treatment and management of infectious diseases viewpoint during the past decades (7) .Cells in such environment may live to 1,000 fold higher concentrations of antifungals than non-biofilm or planktonic cells (8). Nanoparticles can overpower existing drug resistance mechanisms due to their tiny dimensions (9) (range from 10 to 100 nm), so could be considered as an supreme biofilm control strategy. Mainly nanoparticles are categorized into organic and inorganic nanoparticles (10).

In recent times, interest has particularly raised in the case of metal oxide nanoparticles (MNPs) because of, in medical usages as chemical sensing devices, sterilization as antimicrobials fillers, opacifiers, catalysts, semiconductors in the cosmetics industry and microelectronics (11). Fe_3O_4 nanoparticles have gained immense potential in fields of immobilization of bio separation, biomaterials, biomedical and bioengineering application, environmental treatment, and food analysis. All of mentioned potentials are due to the inimitable properties, such as easy separation under external magnetic fields, high surface area, large surface-to-volume ratio, low toxicity, and finally though very important superparamagnetism (12). Furthermore, the use of MNPs as a magnetic fluid hyperthermia inductor can limit the formation of biofilm and decrease cell sticking might also be used in cure of fungal-derived infections (13).

The probable elucidated mechanism of function is that the metal nanoparticles possess positive charges and from the other hand microbes possess negative charges which consequently results in electromagnetic attachment the nanoparticles and the cell surface. So microbes could get oxidized and extinguish immediately (14). In this project, we aimed to study the Fe_3O_4 nanoparticles (SPION), as a nanotechnology, on biofilm formation of *C. albicans*.

MATERIALS AND METHODS

The Fe_3O_4 nanoparticles (SPION) were manufactured by chemical co-precipitation method by the following 1.5 mmol of $FeCl_2 \cdot 4H_2O$ (Merck, Germany) and 3.0 mmol of $FeCl_3.6H_2O$ (Merck, Germany) in 0.01 M HCl solution (Merck, Germany). The solution was rapidly thrown in a 80 ml of 2M ammonia solution (Merck, Germany) stirred uniformly for one hour at room temperature under nitrogen gas. The precipitate washed with absolute ethanol (Merck, Germany) and was separated by using a constant external magnetic field. The procedure was continued, as long as the pH of the output solution gets equal to washing solution pH. Magnetic nanocrystal was then dried at 70°C under vacuum condition (15). Characterization of nanoparticles were checked by FTIR (FTIR, PerkinElmer Frontier) absorption and X-ray diffraction.

Candida albicans PTCC 5207 strain was purchased from the Organization of Iranian Research Organization for Science and Technology (IROST), cultured on Potato Dextrose Agar (PDA) (Scharlau, Spain) and Potato Dextrose Broth (PDB) (Scharlau, Spain) media and incubated at 25°C.

Inoculum preparation was done in polystyrene, flat bottomed, 96- well microplates (JET BIOFIL, Canada). Each well filled with 100 µl of different concentrations of nanoparticles (25, 50, 100, 150, 200, 500, 600, 1000 ppm), 180 µl of PDB and 20µl yeast suspension equal to (0.5

McFarland). Each concentration was inoculated in triplicate. The negative control wells contained only PDB & distilled water. The positive control wells contained PDB, distilled water (concentration 0) and yeast. Microplate incubated at 25°C for 24 hours. This inoculum was used to further tests.

After 24 hours, MIC content measurement on these treatments were carried out using CLSI M27 with some modification (16). MIC described as the lowest concentration of SPION at which ocular preventing the growth of *C. albicans* occurred (17).

The MFC described as the lowest concentration of nanoparticles which produced no measurable growth on the plate (18). This was determined by transferring 10µl of the wells which has over MIC threshold value into PDA plate, then incubated at 25°C for 24 hours to check whether the nanoparticles are fungicidal or not.

Crystal Violet (CV) staining method, which is very simple though with fidelity, was used to biofilm formation measurement. Briefly, after 24 hours of culture, all of the wells were washed three times with 300µl of ringer (Merck, Germany) to delete loosely attached cells. Then, *Candida* biofilms were stabilized with 300µl methanol (Merck, Germany). After 30 min, methanol was thrown away. After exsiccate at room temperature, 150 µl of crystal violet (0.1%) (Merck, Germany) added to the wells and incubated at room temperature. After 5 minutes, wells were softly rinsed under tap water current. The microplate was let to dry at room temperature. Then, 150µl of acetic acid (33% v/v; Sigma-Aldrich, USA) was added to wash out the stain from the wells. Finally, absorbance of each well was read in an ELIZA Reader (BioTek, Elx 800, USA) at 630 nm (19).

The cutoff of optical density (ODC) for the micro-titer plate test was described as three standard deviations higher than the mean OD of the negative control. Isolates were categorized as follows: (20):

ODC = (3 OD of Negative Control)

$OD \leq ODC$ = Non biofilm

$ODC < OD \leq (2 \times ODC)$ = Weak biofilm

$(2 \times ODC) < OD \leq (4 \times ODC)$ = Moderate biofilm

$(4 \times ODC) < OD$ = Strong biofilm

The percentage of biofilm inhibition was computed by following formula (21): Biofilm reduction % = $[((OD_C - OD_B)-(OD_T - OD_B)) / (OD_C - OD_B)] \times 100$

The results were actuarially analyzed using SPSS, Minitab, Excel, Software, Duncan' s test. Samples were considered statistically significant when $p < 0.05$.

RESULTS

Courier tracking in FTIR absorption of SPION can be seen in 581 cm^{-1} area (Fig 1).

Concentration of 100 ppm with no observable colony was determined as MIC. The concentration of 200 ppm was determined as the minimum fungicidal concentration.
The greatest effect of SPION was observed at 200 ppm and highest biofilm formation was seen in concentration of 25 ppm. By increasing the concentration of SPION, their effect on reducing the formation of *Candida albicans* yeast biofilm increased, and this increase was significant up to 200 ppm, but there was no significant difference between 200 and higher concentrations. So, the effective concentration in inhibition biofilm formation was 200 ppm (Fig 2, Table 1)

The percentage of biofilm formation by *C. albicans* was reduced 48.8% at concentration of 25ppm of SPION, and at concentration of 200 ppm of these nanoparticles, no biofilm was formed which can be inferred as 100% inhibition. The type of formed biofilm in concentration of 25 ppm of these nanoparticles was moderate type and by increasing concentration of these nanoparticles, the biofilm formation rate decreased, so that in the concentration of 200 ppm of these nanoparticles, there were no biofilm formation (Table 1).
It was found that the effect of increasing the concentration of SPION on the optical density was interrelated; meaning that increasing concentrations of SPION reduced the optical density of *C. albicans*.

DISCUSSION

As shown in the result, absorption peak at 581 cm^{-1} corresponds to the Fe–O vibration. This absorption peak verifies the synthesis of iron nanocrystals in the magnetite phase (15).
The X-ray diffraction demonstrated that the SPION have an approximately similar size and mean diameter about 70 nm.
Nanoparticles as anti-biofilm agents can destructive systems. The antimicrobial effect of metal nanoparticles has been offered to be because of their high surface area to volume ratio and happens in two stages (22). At first, cationic defects of surface are created by removal of Fe ions and then diffusion of Fe ions in the crystal lattice (23). Since a magnetic field can increase the absorption of nanoparticles into bacterial biofilms, it can be used to eliminate biofilm and also to treat infectious diseases (24, 25). The shape and size of nanoparticles can impress their antimicrobial activity (26). Based on our result, SPION were success in reducing biofilm formation.
It is reported that small particles (sizes 10 - 80 nm) penetrating into the *Escherichia coli* membrane could be the reason why the bacterium should be inactivated by zerovalent iron nanoparticles (Fe^0). Nano-Fe^0 could then react with intracellular oxygen, leading to oxidative stress and finally causing disruption of the cell membrane (27). Since the yeast growth in medium is like a bacterium, and the size of nanoparticles was also within this range (70 nm), then it can be conclude that this nanoparticle size can be of those retardant biofilm formation reasons. Some important nanotoxicities of FeNPs have been therefore detected, such as infusions of cell inflammation mitochondrial damage, detriment to cell livability, reactive oxygen species (ROS), apoptosis, oxidative stress, cell movement impairment, autophagy, and DNA damage (21).

Many of studies have proved metal nanoparticles anti-biofilm effects due to different and common mechanism. Ag-NPs by interaction with the membrane surface of *Candida albicans* and *Saccharomyces cerevisiae* cells, in presence of Ag-NPs, show considerable changes in their

membranes, which are distinguished by the formation of holes called ‘‘pits’’, and eventually, result in cell death (28). Also, Ag-NPs (Silver nanoparticles) can cause apoptotic cell death in *C . albicans* by enhancing of hydroxide (OH^-) ions (29). It is reported that ROS-dependent anticandidal property of ZnO-NPs are in line with ROS production in a concentration-dependent way, as such things can be seen in current study, too (30). Other antimicrobial effects of ZnO nanoparticles include the following: The first is the formation of H_2O_2 on the surface of ZnO-NPs because of the probable hydrogen bond formed between hydroxyl group of cellulose present in fungi with oxygen of ZnO-NPs leading to inhibition of the growth, meanwhile the other is the release of Zn^{2+} ions that is able to damages cell membrane and interacts with intraocular contents (31). Regarding the antifungal effect of Cerium Oxide nanoparticles (CeNP), it is believed that the interaction between cerium and components of the fungal cell wall can afford irreversible changes, such as blocking fungal enzymatic activity (32). It has also been reported that mesoporous TiO_2 Nanoparticles play role in antibiofilm activity due to release of attached bioactive elements (33).

Fe_3O_4 nanoparticles are one of the causative agents of ROS that can prevent growth and infection of *Staphylococcus aureus* (27). It might happen about *C. albicans* though it needed more and detailed studies.

Of more results in accordance with this study about metal nanaoparticles effect on *C. albicans*, is CeNP (Cerium Oxide) antifungal activity on *C. albicans*. As CeNPs concentration of 17 ppm (lowest) yielded a reduction in the livability of the fungus, while a 170 ppm concentration caused the complete prevention of the fungus livability (32). But MIC and MFC of SPION was determined 100 ppm and 200 ppm, respectively. So we can conclude that the antifungal effect of CeNPs is far more than SPION.

In other research, The MIC_{50} value of CuO NPs was determined 1000 ppm for three species of *Candida*. Thus, Nano copper oxide had a weak efficacy on the candida species (26). These results disagree with our results, because SPION had sufficient effect on *C. albicans*. The reason for this can be that different nanoparticles show different effects on the same microbial species.

The MIC of Silver nanoparticles (AgNPs) for the preventing of sessile growth of *C. albicans* ATCC 90028 was 1.5 ppm (34) These results demonstrated the crucially antifungal activity against *C .albicans*; so SPION effect was weaker than AgNPs.

It is observed that antifungal activity was rely strongly on the concentration of ZnO NPs (35), and these results agree with our result, because by increasing in the concentration of SPION, their antifungal and ani-biofilm activity was increased.

In one other research, antifungal effects of 4 types of nano- metal oxides including magnesium oxide (MgO), zinc oxide (ZnO), silicon oxide (SiO_2) and copper oxide (CuO) were surveyed against *C .albicans*. Interestingly, MIC of nano SiO_2 and nano- MgO was higher than 3200 ppm, meanwhile MIC and MFC of nano- ZnO determined as 200 ppm and 400 ppm. Thus, this property for nano-ZnO was greater than nano-CuO; it become clear that, ZnO and CuO nanoparticles possess anti *C. albicans* traits, but while nano-MgO and nano SiO_2 did not have antifungal effect against *C .albicans* at in vitro condition (29). From these results it can be concluded that all of these nanoparticles had a weaker effect compared to SPION against *C. albicans* in our result.

In antifungal effect study of nono- chitosan, by increasing concentration of nano-chitosan particles, biofilm formation rate decreased (36) which is according our results. In our study, in concentration of 200 ppm of SPION 100% *C. albicans* got killed and no biofilm observed. In another research (37), the concentration of 50 ppm of SeNP prevented the formation of biofilms from 60 to 70 percent. In our research, SPION in concentration of 50 ppm was sufficient for 73.05% biofilm inhibition. Therefore SeNP and SPION had almost similar effects on *C .albicans*' biofilm reduction. Although, 5 ppm of AuNPs were significantly successful reducing the metabolic activity of biofilms, and ≥ 20 ppm of AuNPs lead in > 80% reduce (38), AuNPs had more impact on biofilm formation in *C. albicans* than SPION. Researchers reported that SPION were capable to extinguish up to 25% of *Staphylococcus epidermidis* a concentration range of 10– 2000 ppm in a 48 hour old biofilm (39). At concentration of 125 and 250 ppm of ZnO nanoparticles, the biofilm formation *C. albicans* decreased 62% and 85% respectively. Also, it is reported that the concentration of 20 ppm of gold nanoparticles resulted in a 80% reduction in biofilm production of *C. albicans* (4). In our study, at concentration of 100 ppm of SPION, the biofilm formation of C. albicans decreased 87.2% and was completely inhibited at 200 ppm. It is concluding that different nanoparticles show different antimicrobial effects on the same microbial strains. Also it is conclude that SPION act stronger to some nanoparticles and weaker to the others.

Totally in our research, SPION had sufficient efficacy on prevention of *C. albicans*' biofilm formation.

CONCLUSION

In light of the findings of this study, it seems that iron metal oxide nanoparticles (SPION) may be a good candidate of a novel family of fungicidal compounds.

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CONFLICT OF INTREST

All authors certify that they have NO conflict of interest.

FINANCIAL DISCLOSURE

All authors certify that no party having a direct interest in the results of the research supporting the material in the manuscript.

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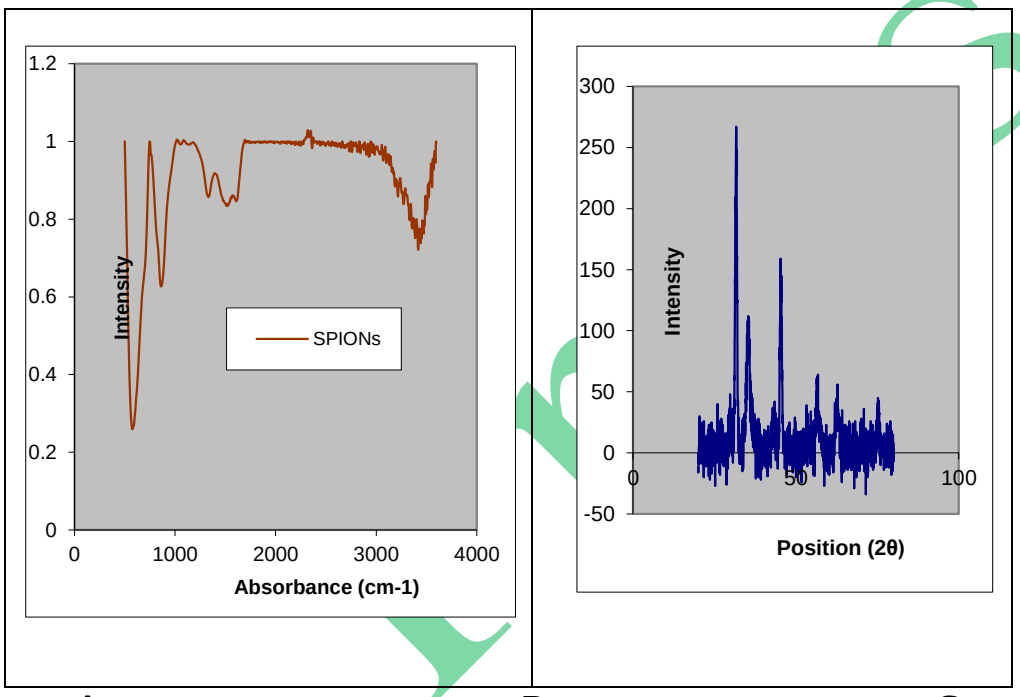

A **B** **C**

Figure 1: (A) courier tracking of SPION, (B) X-ray diffraction of magnetic nanocrystals.

Spring' *pixel* structure in (B) includes: 30.0, 35.6, 43.8, 54.2, 57.2, 62.5 that Miller indices relates to them are (3 1 1) (2 2 0) (5 1 1) (4 2 2) (4 0 0) (4 4 0).

The approximate size of the synthesized nanoparticles is 70 nm and the have cubic spinel structure.

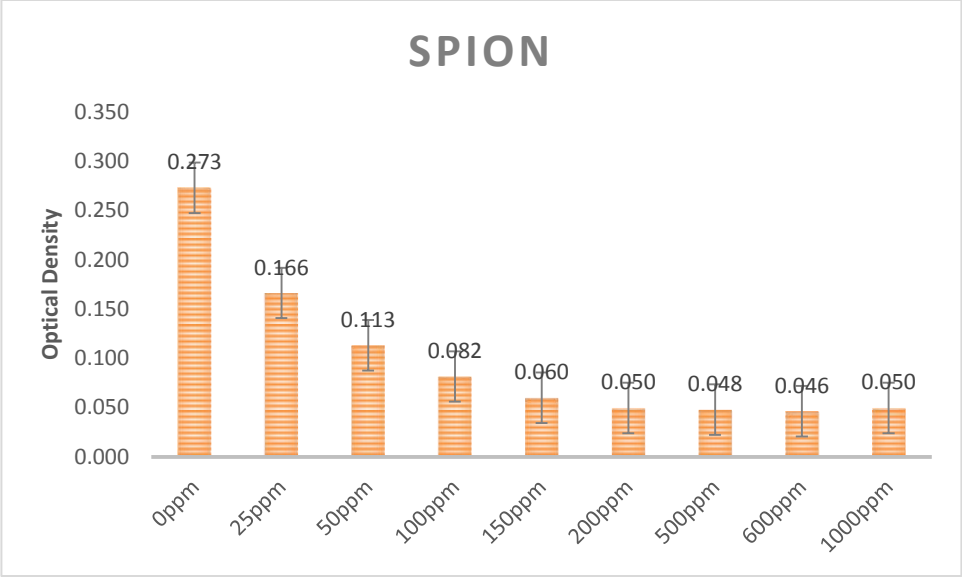

Figure 2: The Effect of SPION on biofilm Formation of *Candida albicans*

Table 1: Effect of SPION Concentration on *Candida albicans* biofilm formation

Nanoparticles	Concentrations (ppm)	Average OD	Standard error	Standard difference	Reduce biofilm formation	Duncan's test	Type of biofilm formation
SPION	0	0.273	0	0	0	G	SB*
	25	0.166	0.003	0.005	48.85	F	MB*
	50	0.11	0.002	0.004	73.05	E	WB*
	100	0.08	0.008	0.014	87.21	D	WB
	150	o.06	0.005	0.009	97.26	C	WB
	200	0.05	0.001	0.002	100	AB	NB*
	500	0.048	0.003	0.005	100	AB	NB
	600	0.046	0.002	0.004	100	A	NB
	1000	0.05	0.001	0.0025	100	AB	NB

SB: strong biofilm formation by *Candida albicans*
MB: Moderate biofilm formation by *Candida albicans*
WB: Weak biofilm formation by *Candida albicans*
NB: Non biofilm formation by *Candida albicans*