Investigating Effects of Superparamagnetic Iron Oxide Nanoparticles on *Candida albicans* Biofilm Formation

Fatemeh Golipour (MSc)
Department of Microbiology, Faculty of Basic Sciences, Hamedan Branch, Islamic Azad University, Hamedan, Iran

Reza Habibipour (PhD)
Department of Microbiology, Faculty of Basic Sciences, Hamedan Branch, Islamic Azad University, Hamedan, Iran

Leila Moradihaghgou (PhD)
Department of Microbiology, Faculty of Basic Sciences, Hamedan Branch, Islamic Azad University, Hamedan, Iran

Corresponding author: Reza Habibipour

Tel: +989183169760

Email: habiby.reza@gmail.com

Address: Department of Microbiology, Islamic Azad University, Hamedan Branch, Hamedan, Iran

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ABSTRACT

Background and Objectives: *Candida albicans* is one of the most common fungal pathogens that can form biofilm, particularly on surface of medical devices. In recent years, *C. albicans* has shown increased resistance to antifungal agents. In this experimental study, we aimed to study effects of superparamagnetic iron oxide nanoparticles (Fe_3O_4 nanoparticles or SPION) on biofilm formation by *C. albicans*.

Methods: First, the SPION were synthesized by chemical co-precipitation. The formation of nanoparticles was confirmed by Fourier-transform infrared spectroscopy and X-ray diffraction. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of SPION were determined. Then, antibiofilm effects of the nanoparticles were investigated by enzyme-linked immunosorbent assay. Finally, data were analyzed using SPSS 22.0 at significance level of 0.05.

Results: According to the results of X-ray diffraction, the SPION had a mean diameter of about 70 nm. MIC and MFC values of SPION against *C. albicans* were 100 ppm and 200 ppm which reduced biofilm formation by 87.2% and 100%, respectively. SPION showed significant inhibitory effects on *C. albicans* growth and biofilm formation.

Conclusion: Based on the findings, SPION may be considered as a novel family of fungicidal compounds. However, further studies are necessary to evaluate the safety of these nanoparticles for treatment of fungal infections in humans.

Keywords: Candida albicans; Biofilms; SPION; Nanoparticles.

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INTRODUCTION

Candida albicans is an opportunistic fungus and a part of the normal gut flora (1, 2). Although the microorganism is not normally harmful, overgrowth of the fungus can result in candidiasis and candidemia (3). The fungus is characterized by multiple virulence factors such as biofilm formation and protease production (4). The main steps of biofilm formation include attachment of fungi to surface, establishment of cellular interactions and formation of colonies (5, 6). In recent decades, biofilms have caused serious challenges in management and treatment of various infectious diseases (7). Compared to non-biofilm forming or planktonic cells, cells within biofilms could survive in an environment containing up to 1,000 fold concentrations of antifungals (8).

The small size (10 to 100 nm) of nanoparticles (NPs) makes them suitable candidates for biofilm inhibition and treatment of drug-resistant infections (10). Recently, much interest has been given to the use of metal oxide NPs (MNPs) in various industries (11). Particularly, Fe_3O_4 NPs have emerged as multi-potential agents with notable properties including simple separation under external magnetic fields, high surface area, large surface-to-volume ratio and low toxicity (12). Furthermore, the use of MNPs as magnetic fluid hyperthermia inductors could limit biofilm formation and may be used for treatment of resistant fungal infections (13). Evidently, electromagnetic attachment between positively charged metal NPs and the negatively charged microorganisms and the subsequent oxidization could lead to immediate elimination of the microorganisms (14). In this study, we aimed to investigate effects of Fe_3O_4 NPs (SPION) on biofilm formation by *C. albicans*.

MATERIALS AND METHODS

Fe_3O_4 NPs (SPION) were prepared by chemical co-precipitation of 1.5 mmol $FeCl_2 \cdot 4H_2O$ (Merck, Germany) and 3.0 mmol $FeCl_3.6H_2O$ (Merck, Germany) in a 0.01M HCl solution (Merck, Germany). The solution was rapidly mixed with 80 ml of 2M ammonia solution (Merck, Germany) and stirred uniformly for one hour at room temperature under nitrogen gas. The precipitate was washed with absolute ethanol (Merck, Germany) and separated using a constant external magnetic field. The procedure continued until the pH of the output solution matched the pH of the washing solution. Magnetic nanocrystals were *dried* at *70 °C under vacuum conditions* (15). Nanoparticles were characterized by *Fourier-transform infrared spectroscopy* (FTIR, PerkinElmer Frontier) and X-ray diffraction.

C. albicans PTCC 5207 was purchased from the 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 for 24 hours.

Inoculum preparation was done in polystyrene, flat bottom 96-well microplates (JET BIOFIL, Canada). Each well was filled with 100 µl of different concentrations of NPs (25, 50, 100, 150, 200, 500, 600, 1000 ppm), 180 µl of PDB and 20 µl of a yeast suspension equivalent to 0.5 MacFarland standard. Each concentration was inoculated in triplicate. The negative control wells contained only PDB and distilled water. The positive control wells contained PDB, distilled water (concentration 0) and yeast. The microplates were incubated at 25 °C for 24 hours. This inoculum was used for future testing. Minimum inhibitory concentration (MIC) was determined according to the CLSI M27 guidelines with some modification (16). MIC was described as the lowest concentration of SPION that prevented the growth of *C. albicans* (17). Minimum fungicidal concentration (MFC) was described as the lowest concentration of NPs which produced no measurable growth on the plate (18). The MFC was determined by transferring 10 µl from wells with MIC values over the threshold value into the PDA plate, followed by 24 hours of incubation at 25 °C.

Crystal violet staining was carried out to evaluate biofilm formation. Briefly, after 24 hours of culture, all wells were washed trice with 300 µl of ringer (Merck, Germany) to remove loosely attached cells. Then, 300 µl of methanol (Merck, Germany) were used to stabilize *Candida* biofilms. After 30 min, methanol was aspirated at room temperature, 150 µl of 0.1% crystal violet (Merck, Germany) were added to the wells, and the microplate was incubated at room temperature for 5 min. Next, the wells were softly rinsed under running tap water and dried at room

temperature. Next, 150 µl of acetic acid (33% v/v; Sigma-Aldrich, USA) were added to wash out the stain from the wells. Finally, absorbance of each well was read at 630 nm in an ELISA Reader (BioTek, Elx 800, USA) (19). The optical density cut off (ODC) for the microtiter-plate test was defined as three standard deviations above the mean OD of negative controls (20). Accordingly, the isolates were categorized as non- ($OD \leq ODC$), weak- [$ODC < OD \leq (2 \times ODC)$], moderate- [$(2 \times ODC) < OD \leq (4 \times ODC)$] and strong- [$(4 \times ODC) < OD$] biofilm forming.

The percentage of biofilm inhibition was calculated based on the following formula (21): Percent biofilm inhibition = $[(OD_C - OD_B)-(OD_T - OD_B) / (OD_C - OD_B)] \times 100$. In the formula, OD_C, OD_B, and OD_T represent OD of negative control, positive control (blank) and treatments, respectively. Statistical analysis of data was carried out using SPSS 22.0 and Microsoft Excel 2010 at significance level of 0.05.

RESULTS

SPION FTIR absorption spectrum showed a band at 581 cm^{-1}, which corresponded to the Fe-O vibration (Figure 1). 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 of about 70 nm.

Concentrations of 100 ppm and 200 ppm were determined as the MIC and MFC, respectively. The highest level of biofilm formation was observed at 25 ppm. As expected, increasing the concentration of SPION to 200 ppm significantly increased the biofilm inhibition activity against *C. albicans*. Furthermore, the most effective concentration for inhibition of biofilm formation was 200 ppm (Figure 2 and Table 1).

We recorded a 48.8% reduction of biofilm formation by *C. albicans* at 25ppm of SPION, while no biofilm (100% inhibition) was formed at 200 ppm of SPION.

Figure 1- (A) FTIR analysis of SPION. (B) X-ray diffraction patterns of magnetic nanocrystals.

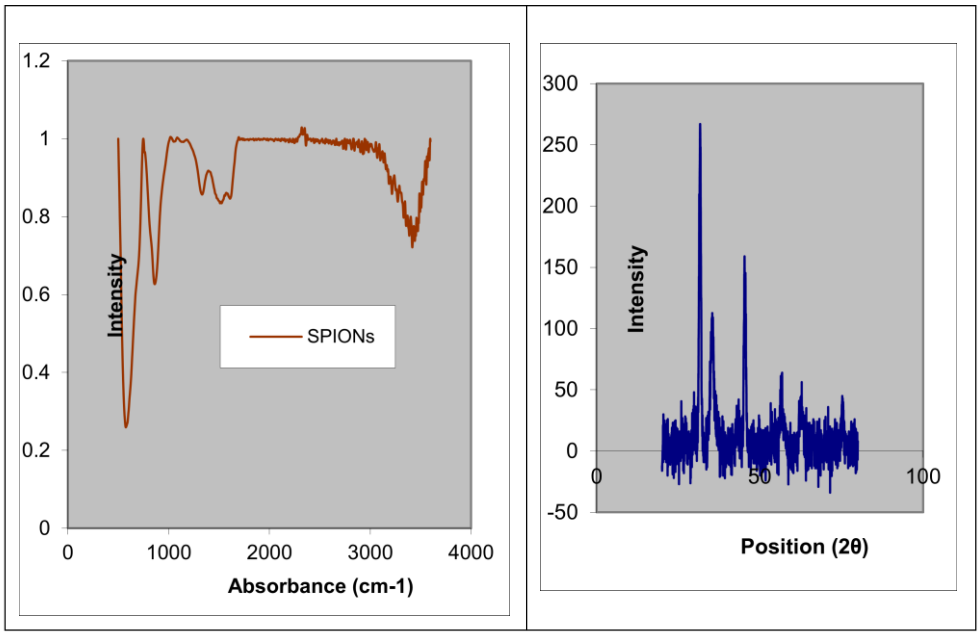

Figure 2- Effects of SPION on biofilm formation by *C. albicans*

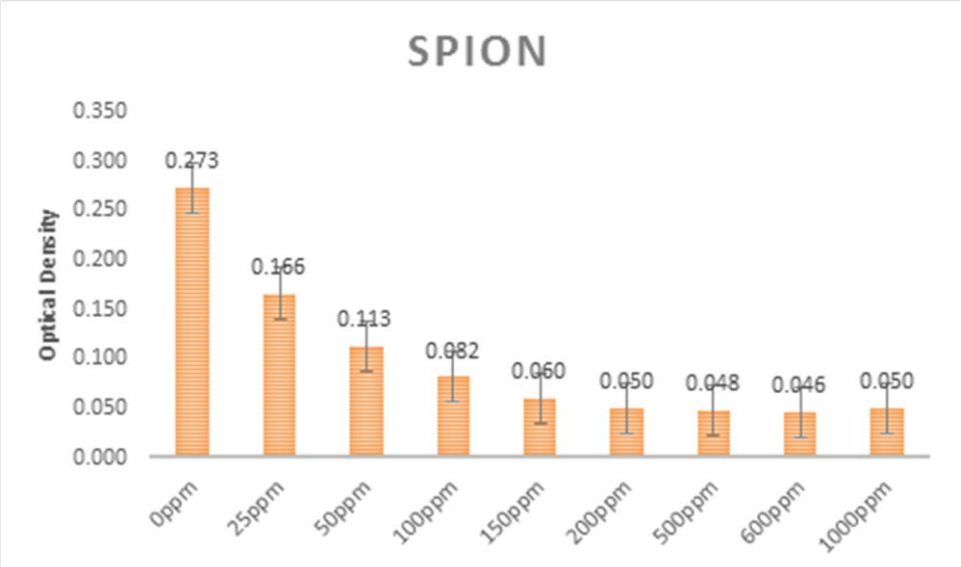

Table 1- Effects of various concentrations of SPION on biofilm formation by *C. albicans*

Nanoparticles	Concentration (ppm)	Average optical density	Standard error	Standard deviation	Biofilm reduction	Duncan's test	Biofilm formation ability
SPION	0	0.273	0.000	0.000	0	f	Strong
	25	0.166	0.003	0.005	48.85	e	Moderate
	50	0.11	0.002	0.004	73.05	d	Weak
	100	0.08	0.008	0.014	87.21	c	Weak
	150	0.06	0.005	0.009	97.26	b	Weak
	200	0.05	0.001	0.002	100	ab	None
	500	0.048	0.003	0.005	100	ab	None
	600	0.046	0.002	0.004	100	a	None
	1000	0.05	0.001	0.002	100	ab	None

DISCUSSION

The antimicrobial effect of metal NPs is related to their high surface area to volume ratio (22). Interestingly, during the synthesis of FeNPs, produced colloidal magnetite can be easily oxidized in acidic suspensions in two steps: cationic defects are created on the surface by reduction of Fe^{2+} ions and then diffusion of Fe^{3+} ions takes place in the crystal lattice (23). Since a magnetic field can increase the absorption of NPs into bacterial biofilms, it can be used to eliminate biofilms as well as to treat infectious diseases (24, 25). The shape and size of NPs can influence their antimicrobial activity (26). It is reported that penetration of zerovalent iron NPs (Fe^0) sized 10-80 nm into *Escherichia coli* membrane could lead to bacterial growth inhibition by interacting with intracellular oxygen, thereby causing oxidative stress and disruption of the cell membrane (27).

The antibiofilm effects of metal NPs have been demonstrated in numerous studies. Silver NPs can interact with the membrane surface of *C. albicans* and *Saccharomyces cerevisiae* and form ‘‘pits’’ that eventually result in cell death (28). These NPs can also cause apoptotic cell death in *C. albicans* by enhancing hydroxide ions (29). It has been reported that reactive oxygen species (ROS)-dependent anticandidal property of ZnO NPs is due to the production of ROS in a concentration-dependent manner (30). Regarding the antifungal effect of cerium oxide NPs (CeNP), it is believed that the interaction between cerium and components of the fungal cell wall can cause irreversible changes, such as blocking fungal enzymatic activity (31). It has also been reported that the antibiofilm activity of mesoporous TiO_2 NPs could be due to release of attached bioactive elements (32). It is also thought that the

presence of Fe_3O_4 NPs result in ROS production, which consequently prevents *Staphylococcus aureus* growth (27). A study on effects of CeNPs on *C. albicans* reported that 17 ppm (lowest) CeNP could reduce the viability of the fungus, while concentration of 170 ppm completely inhibited the growth of *C. albicans* (32). Given the obtained MIC and MFC values for SPION in our study, we can conclude that the antifungal effect of CeNPs against *C. albicans* is far more than that of SPION. In another study, the MIC_{50} value of CuO NPs was 1000 ppm against three *Candida* species (26), which indicates that these NPs have weaker antifungal activity compared to SPION. The MIC of AgNPs for preventing growth of sessile *C. albicans* ATCC 90028 was 1.5 ppm (33), which is significantly less than the MIC value obtained from SPION. In our study, increasing the concentration of SPION increased the antifungal and antibiofilm activity of the NPs, which has been also observed in other studies (34, 35). In a study, antifungal effects of four MNPs including magnesium oxide (MgO), zinc oxide (ZnO), silicon oxide (SiO_2) and copper oxide (CuO) were investigated against *C. albicans*. Interestingly, MIC of nano-SiO_2 and nano-MgO was higher than 3200 ppm, while MIC and MFC of nano-ZnO were 200 ppm and 400 ppm, respectively (29).

In our study, 200 ppm of SPION completely inhibited biofilm formation and growth of *C. albicans*. In a previous study, 50 ppm of SeNP prevented biofilm formation by 60 to 70% (36), which is similar to the antibiofilm effects produced by the same concentration of SPION. In another study, AuNPs at concentrations ≥ 20 ppm could inhibit metabolic activity of biofilms more than 80% (37).

A concentration range of 10-2000 ppm SPION was capable of eliminating up to 25% of *Staphylococcus epidermidis* present in a 48-hour old biofilm (38). At concentrations of 125 and 250 ppm, ZnONPs could decrease biofilm formation by *C. albicans* by 62% and 85%, respectively (4).

CONCLUSION

Based on the findings, SPION could be considered as a novel family of fungicidal compounds.

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CONFLICT OF INTREST

All authors certify that there is no conflict of interest.

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