Antifungal Activity of TiO$_2$ nanoparticles and EDTA on *Candida albicans* Biofilms

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**Abstract**

**Aim of Study:** Biofilm of *Candida albicans* signify as a complex cellular congregation with major implication in pathogenicity. This kind of lifestyle of fungus as a biofilm can inhibit immune system function and antifungal therapy, thus complicates the treatment of infectious diseases, particularly in field of chronic infections associated with medical devices. In this study effects of Titanium dioxide (TiO$_2$) nanoparticles and Ethylene Diamine Tetra Acetic Acid (EDTA) were evaluated on *C. albicans* biofilm by using the different techniques.

**Methods:** TiO$_2$ nanoparticles were synthesized from precursor Titanium tetrachloride (TiCl$_4$). To assay biofilm formation ability of yeast cells, *C. albicans* strains (ATCC10231 and ATCC76615) were grown in a flat-bottom 96-well microtiter plates and antifungal effects of TiO$_2$ and EDTA were evaluated on *C. albicans* biofilms using ATP bioluminescence and tetrazolium salt (XTT) reduction assays. Furthermore, morphology of biofilms after 48 h was observed by scanning electron microscopy (SEM).

**Results:** Synthesized TiO$_2$ nanoparticles and EDTA had effective antifungal properties at the concentration of 5.14, 8.09 µg/ml for fluconazole susceptible strain and 5.35, 11.33 µg/ml for fluconazole resistant strain of *C. albicans* biofilms compared to fluconazole drug ($P < 0.05$).

**Conclusion:** Using TiO$_2$ nanoparticles can be considered as a new agent in field of prevention of fungal biofilms especially biofilms formed on the surface of medical devices.

**Keywords:** Antifungal agent, Biofilm, *Candida albicans*, Nanoparticles, TiO$_2$.

**Introduction**

*Candida albicans* (*C. albicans*) is one of the most important opportunistic fungi that cause different kind of diseases from superficial to systemic infections under favorable conditions (Weber *et al*. 2008). Currently *C. albicans* has more effective role than other nosocomial pathogens. This fungus has proper potential for biofilms formation (Kojic and Darouiche 2004). Cells in the biofilms exhibit an increase resistance to the antifungal drugs. Biofilm on dentures are an important medical problem as well on catheters which provides a common infection for hospital patients (Jabra-Rizk *et al*. 2004; Kojic and Darouiche 2004). Titanium dioxide (TiO$_2$) is a white powder with melting
temperature of 550 °C and dissolution in hot concentrated nitric acid. Application and effectiveness of titanium dioxide are related to the crystal structure, shape and size. The selection of TiO2 nanoparticles for this study is because of its unique features including: high chemical resistance, non-toxic, long lasting nature, availability and low cost (Gao et al. 2004; Enyashin and Seifert 2005; Liao and Liao 2007).

Using a novel method to inhibit attachment of cells to the surface and eliminate of fungal mass over surfaces is a valuable way to control infections (Butterfield et al. 2002). Nowadays great growing of nanotechnology in different branches of science such as engineering and medical is obviously noticeable. In recent years, nanomaterials have considerable acceptance to use as an antimicrobial effect due to different physical, chemical and electrical properties in ultra tiny form that unavailable in larger forms (Zhang et al. 2008; Jiang et al. 2009).

In this study we evaluated antifungal effect of TiO2 nanoparticles and Ethylene Diamine Tetra Acetic Acid (EDTA) as a chemical agent on fluconazole susceptible and resistant standard strains of C. albicans strains. We used C. albicans biofilm model according to the role of this fungus as an important nosocomial infection and also capability of this microorganism to adhere to many surfaces and forming biofilms.

Materials and Methods
Preparation of TiO2 nanoparticles
TiO2 nanoparticles were synthesized through the hydrolysis of Titanium Tetrachloride (TiCl4) as precursor. In this step TiCl4 was slowly added into the 58 ml distilled water under constant string for 5 hours. The solution was aged for 24h at ambient temperature. Then gel was dried in oven for 12h at 60° C and finally calcinated at the 550° C (Sasirekha et al. 2009).

Characterising of TiO2 nanoparticles
To access information of shape and estimating size of nanoparticles, SEM (Philips) micrograph was taken. To identifying type of TiO2 nanoparticles, the synthesized TiO2 powder was characterized by X-ray diffraction (XRD) technique (XPERT; model 95) with λ = 1.54178 Å (Haghighi et al. 2012).

Preparation of standard fungal cell suspension
Fluconazole-susceptible C. albicans (ATCC 10231) was obtained from department of mycology, faculty of veterinary medicine, Tehran university and fluconazole-resistant standard strains of C. albicans (ATCC 76615) was purchased from Shanghai, China pharmaceutical university army, which were used for biofilm formation and the antifungal activity of mentioned agents. First, two strains were grown on sabouraud dextrose agar medium (SDA Merck, Germany) at 37°C for 18h. Then, freshly colonies were inoculated into yeast nitrogen base medium (YNB medium; Himedia Co) containing 50 mM glucose and incubated at 37°C for 24 hours. After that, a few colonies of the yeasts were transferred into a test tube containing sterilized PBS with pH: 7.2. The turbidity of suspension of cells was compared to 0.5 McFarland standard to estimate cell density and finally yeast cells were counted and adjusted at 1× 10^6 cells/ml by Neubauer slide (Rex et al. 2008; Rodriguez-Tudela et al. 2008).

Biofilm formation
Candida biofilms were grown on 96-wells plates. First, 100µl YNB medium was enriched with 50 mM glucose. Then, 10µl of C. albicans cells was added to each well. The yeast cells were allowed to adhere to the bottom of the wells for 90 min at 37°C. Next, content of each well was gently submerged in 100µl PBS and then added 200µl YNB medium with 50 mM glucose. The plates were incubated at 37°C for 48 h (Ernst and Rogers 2005; Kavanagh 2007). Later, the YNB medium was removed and washed by PBS and according to our
pervious study different concentration of TiO2 (4-8 µg/ml) and EDTA (6.5-15 µg/ml) were added to each well separately. Finally volume of wells was reached to 100µl with RPMI-1640 (Gibco).

**XTT assay**

XTT [2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5- (phenyl amino) carbonyl]-2H-tetrazolium hydroxide], as colorimetric assay was carried out to assay antifungal effect of the nanoparticles. This method is based on determining the viability of collected cells. Plates were incubated with 50µl of the YNB medium with 50 mM glucose, 100 µl XTT (Sigma) and 50µl coenzyme Q0 (Sigma). Plates were incubated at 37°C for 3 h. Optical absorbance was measured at the wavelength of 492 nm by an ELISA reader (Awareness Technology Co) (Ernst and Rogers 2005; Kavanagh 2007).

**ATPase assay**

ATP is a marker for cell viability. Because of its presence in all metabolically active cells, the concentration declines very rapidly when the cells undergo the biocides. The ATPase assay is based on production of light at 560 nm through the reaction of ATP with added luciferase and D-luciferin (Case 2001). After biofilms formation and treatment of cells by the nanoparticles, ATPase assay was used to confirm the results. For this assay, 10 µl of susceptible and resistant C. albicans biofilms and 10 µl complex reagent containing luciferin, luciferase, and Mg2+ mixed in each well of Luminescence microplates. Then optical density was measured by luminometer (Berthold Co) at 560 nm.

**Statistics studies**

Data analysis was performed by t-test method with SPSS Statistics version 17. The level of statistical significance was set at P<0.05.

**Fig.1.** Biofilm formations of C. albicans (ATCC10231 and ATCC76615) in YNB medium containing 50 mM glucose in the absence and the presence of TiO2 nanoparticles, EDTA and Fluconazole at effective concentration. The methods used for biofilm quantification were (a) XTT reduction assay and (b) ATP bioluminescence assay. Data were means ± standard deviations of three independent experiments (P < 0.05).
Synthesized TiO₂ nanoparticles were regular with monotonous forms. Also we observed that the diameter of the TiO₂ nanoparticles were on average 70-100 nm by SEM and XRD.

The type of crystalline structure of the synthesized TiO₂ nanoparticles have been considered by means of XRD with Cu Ka radiation at wavelength λ = 1.54178 Å, and measurements were performed using a θ–2θ goniometry (Haghighi et al. 2012).

In this study, TiO₂ nanoparticles at the concentration of 5.14 µg/ml and EDTA at the concentration of 8.09 µg/ml inhibited biofilms of susceptible-fluconazole strain of *C. albicans* which was shown by using XTT and ATPase assays. TiO₂ at 5.35 µg/ml and EDTA at 11.33 µg/ml stopped growth of resistant-fluconazole strain of *C. albicans*. Fluconazole is one of the most effective drugs that inhibited *C. albicans* growth used as a control in this study. Results showed that at the concentration of 4 and 8µg/ml, fluconazole stopped *C. albicans* biofilm for susceptible and resistant strains, respectively (Fig. 1).

ATPase assay results showed that the concentration of the mentioned antimicrobial agent against biofilms was according to XTT assay. Synthesized TiO₂ nanoparticles and EDTA suppressed *C. albicans* biofilms at the concentration of 5.14, 8.09 µg/ml for fluconazole susceptible strain and 5.35, 11.33µg/ml for fluconazole resistant strain. Although, in comparison of control groups differences were more than the results of XTT method.

All tests were performed in three independent experiments (P < 0.05).

**Discussion**

Attachment of micro organisms to a surface and embedding the cells inside the polymeric substances cause a community named biofilms which can significantly cause a major infection. *Candida* is an important human fungal flora causing nosocomial infection. *Candida* species have capability to adhere to many surfaces and form biofilm structure (Wesenberg-Ward et al. 2005). Recent evidences have revealed that in more than 65% of microbial infections, biofilms have critical role (Ferreira et al. 2009). Increasing fungal infections and the significant health equipments problems associated with immunocompromised disorder hosts such as AIDS and cancers, hematological disorders are required to prevent the biofilms formation or eliminate them from surfaces (Williams et al. 2011). Many researchers investigated the removal of fungi using variety of conventional drugs and disinfectants such as antifungal biocides or UV radiation to eliminate fungi from surface and from aqua environments but most of them show general drug resistance and environmental hazards (Theraud et al. 2004).

These days understanding of antimicrobial properties of nanoparticles have been attracted interests to search a new strategy for controlling disease by preventing at elementary stage and inhibition of spreading of infection (Habimana et al. 2011). According to some studies (Seven et al. 2004; Lonnen et al. 2005), nanoparticles such as TiO₂ have antimicrobial efficacy which could be considered as a self-cleaning agent. Akiba and colleagues evaluated antifungal effects of a tissue conditioner coating agent with TiO₂ photocatalyst that found reduction in the number of viable *C. albicans* cells after 60 minutes UV light exposure significantly (Akiba et al. 2005).

Numerous investigators have shown that conventional antifungal therapy, such as the azoles especially fluconazole, are associated with ability to elimination of Candida organisms embedded in biofilm (Uppaluri et al. 2011). For this reason fluconazole was used as a positive control for determining the antifungal ability of TiO₂ nanoparticles and EDTA.

The maximum elimination of *C. albicans* by TiO₂ and EDTA was obtained in concentration of 5.14, 8.09 µg/ml for susceptible fluconazole strain (ATCC10231) as well as 5.35, 11.33 µg/ml, for resistant fluconazole strain (ATCC76615), respectively. The results
indicated that viability of biofilms decrease with increasing TiO2 concentration as well as EDTA. We observed suitable results in comparison to fluconazole as a potent drug.

Yeast cells of C. albicans due to possess thick cell wall consist of glucan and chitin are more resistant than bacteria. It was reported that TiO2 nanoparticles by producing intracellular reactive oxygen species (ROS) induce destructive effects inside the microbial cells, oxidation of intra cellular Coenzyme A and peroxidation of the plenty of lipids which decrease respiratory activity and subsequently cause death cell (Battin et al. 2009; Foster et al. 2011).

Previously in vitro studies (Raad and colleagues 2008) have shown that the EDTA is a chelating agent can restrict C. albicans biofilm formation through inhibitory of its filamentation. Further studies showed that EDTA could interrupt microbial biofilm through its chelating of iron, calcium and magnesium and several essential components that associated to biofilm matrix (Raad et al. 2008). Despite these features, EDTA has some side effects such as burning sensation and dermatitis at the puncture site which limits its use (Lamar 1964).

Biofilms inhibition could be done by different materials (Dunne 2002). This study showed that TiO2 not only able to kill C. albicans with increasing concentration but also can inhibit C. albicans biofilm formation at the less concentration than EDTA. By using XTT technique, antifungal effect of the components can be assayed accurately which was shown in our results (Berridge et al. 1996).

Results of ATPase assay were in agreement with XTT method. The major advantages of ATPase assay compared to conventional methods for viability detection are high sensitivity, excellent linearity, simplicity, fast results with lack of cell harvesting and separation steps. Furthermore this method requires less time for analyzing without need to further incubation (Pihlasalo 2011). Consequently in field of controlling and eliminating of C. albicans biofilms, TiO2 nanoparticles can be considered as an alternative antifungal, although further investigation is necessary to evaluate the toxicity of these particles.

It can be concluded that XTT technique and ATPase assay both can be used to study antibiofilm effects of nanoparticles and biocides although ATPase assay achieve more reliable results in less consuming time.

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References


