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Short Report

Extracellular Production of Silver Nanoparticles by Using Three Common Species of Dermatophytes: *Trichophyton rubrum, Trichophyton mentagrophytes* and *Microsporum canis*

Maryam Moazeni[1], Niloofar Rashidi[1], Ahmad R. Shahverdi[2], Fatemeh Noorbakhsh[3] and Sassan Rezaie[*1,4]

[1]*Div. of Molecular Biology, Dep. of Medical Mycology and Parasitology, School of Public Health, Tehran University of Medical Sciences, Tehran;* [2]*Dep. of Pharmaceutical Biotechnology and Medical Nanotechnology Research Center, School of Pharmacy, Tehran University of Medical Sciences, Tehran;* [3]*Dep. Of Biology, Islamic Azad University, Varamin-Pishva Branch, Varamin; Dept. of Medical Biotechnology, School of Advanced Medical Technologies, Tehran University of Medical Sciences, Tehran, Iran*

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ABSTRACT

Background: To develop a new green approach for biosynthesis of silver nanoparticles, myconanotechnology has been represented as a novel field of study in nanotechnology. In this study, we have reported the extracellular synthesis of highly stable silver nanoparticles using three species of dermatophytes: *Trichophyton rubrum*, *Trichophyton mentagrophytes* and *Microsporum canis*. **Methods:** Clinical strains of these species were grown in a liquid medium containing mineral salt and incubated at 25°C for 5-7 days. The cell-free filtrate of each culture was obtained and subjected to synthesize silver nanoparticles in the presence of 1 mM $AgNO_3$. **Results:** The reduction of Ag+ ions in metal nanoparticles was investigated virtually by tracing the solution color which was switched into reddish-light brown after 72 h. For *T. mentagrophytes*, a UV-visible spectra demonstrating a strong, quite narrow peak located between 422 and 425 nm was obtained. For *M. canis,* a fairly wide peak centering at 441 nm and for *T. rubrum,* a weak spectrum to decipher were observed. According to transmission electron microscopy (TEM) results, fairly uniform, spherical, and small in size with almost less than 50 nm particles were forms in case of *T. mentagrophytes*. For the other two species, TEM images showed existence of small spherical nanosilvers but not as small as nanoparticles synthesized by *T. mentagrophytes*. **Conclusion:** We observed that species belong to a single genus of the fungi have variable ability to synthesize silver nanoparticles extracellulary with different efficiency. Furthermore, the extracellular synthesis may make the process simpler and easier for following processes. *Iran. Biomed. J. 16 (1): 52-58, 2012*

Keywords: Nanoparticles, Dermatophytes, Extracellular biosynthesis

INTRODUCTION

The unique size-dependent characteristics of nanoparticles make them indispensable in many areas of human life ranging from industries like aerospace engineering to natural science, such as medicine, biology and food technology [1]. In aspect of medicine, nanobiotechnology poses an upcoming and rapid developing field with various kinds of applications especially for human therapeutics [2].

Apart from antimicrobial properties, anti-cancer effect of nanoparticles has been recently documented [1] as well as anti-fungal and anti-tumor drugs using simple delivery systems have already been marketed. Moreover, successful application of nanoparticles in drug delivery and controlled-drug release has been newly reported [3, 4]. Hence, understanding of biological process in nanoscale level is a strong driving force behind development of nanotechnology [5].

In very recent years, the mycosynthesis of metal nanoparticles or myconanotechnology has drawn the attention of researches to the novel field of

[*]Corresponding author; Tel.: (+98-21) 8895 1392; Fax: (+98-21) 6646 2267; E-mail: srezaie@sina.tums.ac.ir

biosynthesis of nanomaterials. This new field of nanoscience is at the interference of nanotechnology and mycology, comprising an interesting new applied science with considerable potentials owing to the wide range of diversity of fungi [6].

In the world of modern nanoscience which toxic synthesis protocols are being used to obtain the nanoparticles, there is a need to evolve a green approach for nanomaterial synthesis. Therefore, eco-friendly, non-toxic approaches for biosynthesis of nanomaterials and nanostructures have been newly emerged as a promising method to meet the need [7-9]. With respect to this fact, there has been an increasing tendency for re-exploring of microorganisms as potential biofactories for synthesis of functional metallic nanoparticles. The fungal systems or myconanofactories have already been exploited for the biosynthesis of metal nanoparticles of silver, gold, zirconium, silica, titanium, iron (magnetite) and platinum [6] as well as ultrafine oxide nanoparticles, such as Sb_2O_3 [10, 11] and TiO_2 [11].

A large number of fungal strains are capable to synthesize silver nanoparticles (SNP) extracellulary, among which *Fusarium oxysporum* [12], *Bacillus licheniformis* [13], *Aspergillus fumigatus* [14], *Aspergillus niger* [15], *Aspergillus clavatus* [16], *Penicillium brevicompactum* [17], *Cladosporium cladosporioides* [18], *Fusarium semitectum* [19], and yeast strains MKY_3 [20] have been previously described.

As a matter of fact, fungi have some advantages over the bacteria and plants. Fungal mycelia are able to resist the flow pressure, agitation, high temperature and other inappropriate conditions. Furthermore, the extracellular secretion of reductive agents makes the downstream processing easier and finally the mycosynthesized nanoparticles can be directly used in various applications due to the absence of unnecessary cellular components into the experimental solution [21].

Dermatophytes are considered as a real potent skin parasite in public health as to induce superficial skin, hair and nail infections. However, the highly useful utilization of them should not be ignored in nanomycotechnology.

In the present investigation, for the first time, we have reported the extracellular synthesis of highly stable SNP using three more common species of dermatophytes including *Trichophyton rubrum*, *Trichophyton mentagrophytes* and *Microsporum canis.*

MATERIALS AND METHODS

Fungal strains. Three strains of dermatophytes, *T. rubrum, T. mentagrophytes* and *M. canis,* were used in this investigation. The strains were isolated from cutaneous specimens of patients who were referred to Medical Mycology Laboratory, Department of Medical Mycology, Tehran University of Medical Sciences, Iran. The isolates were grown on Sabouraud dextrose agar medium (SDA) and preserved at 25°C until use.

Preparation of cell-free fungal filtrate. The liquid medium containing mineral salts was used to make an efficient medium for fungal growth and establish a biomass competent for SNP biosynthesis. The medium contained (g/l): KH_2PO_4 7.0, K_2HPO_4 2.0, $MgSO_4.7H_2O$ 0.1, $(NH_4)_2SO_4$ 1.0, yeast extract 0.6, and glucose 10.0 [16]. The components were mixed completely by stirring on a magnetic stirrer and then the solution was boiled to digest the undissolved substances and filtered through filter paper. Three Erlenmeyer flasks each containing 200 ml of the mentioned medium were inoculated with three testing strains: *T. rubrum, T. mentagrophytes* and *M. canis.* The three flasks were incubated aerobically on an orbital shaker at 25°C agitating at 150 rpm. Since the growth rate of dermatophytes is low for getting enough amount of biomass, the flasks were incubated under the mentioned conditions for two weeks. Eventually, 2.5, 0.5 and 1.5 g of biomasses were obtained after this period of time for *T. rubrum, T. mentagrophytes* and *M. canis*, respectively. Afterward, the biomasses were extensively washed twice with sterile distilled water and then harvested by sieving through a plastic sieve, followed by extensive washing to remove all medium ingredients. Fresh and clean biomasses were taken into three new flasks containing Milli-Q deionized water with the ratio of 1:10. Then, they were agitated at the same condition as described above but for 72 h. Cell-free filtrates were obtained by utilizing Whatman filter paper No. 1.

Synthesis of silver nanoparticles. Cell-free filtrates were treated with $AgNO_3$ in such a way that a solution with 1 mM of AgNO3 in final concentration was made. The flasks were agitated at 150 rpm in dark. A negative control (only filtrates, without the silver ion) was also run along with the experimental flasks.

Characterization of synthesized SNP. Aliquots of 1ml of the reaction solutions were removed after 72 h and consequently subjected to UV-visible spectroscopy (Labomed Inc., USA). The absorbance was measured at a resolution of 1 nm. The shape and also estimated size of the SNP were determined by transmission electron microscopy (TEM). TEM images of the samples were obtained using a transmission electron microscope (Philips 30ML20, The Netherlands) at a voltage of 100 kv.

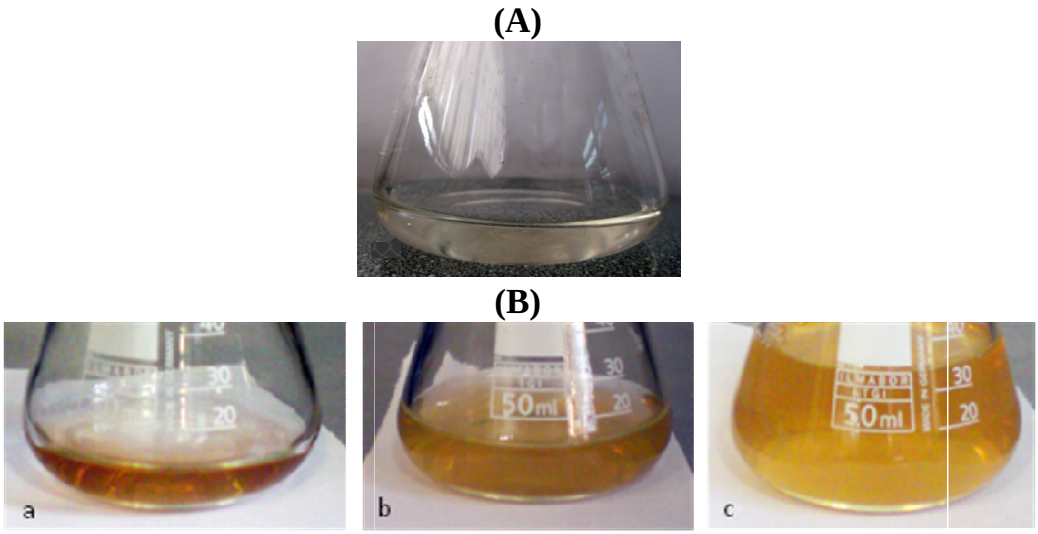

Fig.1. Comparison between the color of the solution before and after $AgNO_3$ addition. **(A)** Cell filtrate at the beginning of the reaction; **(B)** cell filtrates of (a) *T. mentagrophytes*, (b) *M. canis* and (c) *T. rubrum* with silver ion (1 mM) after 72 h of reaction.

Evaluation of Antifungal activity of synthesized SNP. In order to assess the antimicrobial effect of synthesized silver nanoparticles, *Candida albicans* standard strain (ATCC 10261) was applied. The isolate was kept at -80°C as 20% glycerol stocks and were subcultured, as required, on SDA plates at 30°C. Microdilution antifungal susceptibility testing of the *C. albicans* isolate was performed by the broth microdilution method described in CLSI document M27-A3 [22] and performed with SNP and fluconazole as a reference antifungal agent. Silver nanoparticles synthesized by *T. mentagrophytes* were applied as the representative of the nanoparticles produced by three strains. RPMI 1640 medium with L-glutamine and phenol red without bicarbonate and buffered to pH 7.0 with 0.165 mol/L MOPS (3-(N-morpholino) propanesulfonic acid).

Fluconazole was dissolved in sterile diluted water to 64 µg/L, and then diluted to the final concentration of 0.0125 16 µg/L with the medium according to the standard in the Clinical and Laboratory Standards Institute (CLSI) reference method. After growing on SDA (Merck, Germany) at 35°C for 24 h, the yeasts were re-suspended in culture medium, adjusted with a spectrophotometer at 530 nm wavelength and then diluted in culture medium to prepare a working solution at a concentration of 2.5×10^3 cells/mL and incubated at 35°C for 48 h. All tests were carried out in duplicate. The interpretive criteria for susceptibility to fluconazole were published by the CLSI [22], and were as follows: (i) for fluconazole, susceptible, ≤ 8 µg/ml; susceptible dose-dependent, 16 to 32 µg/ml; and resistant, ≥64 µg/ml. For silver nanoparticles, a solution of 16 µg/L was prepared and diluted to the final concentration of 0.0125-16 µg/L with the medium. The same inoculum size was applies for SNP susceptibility test. The 96-well plate was incubated at 35°C for 48 h.

RESULTS

Silver reduction. Changing in color of the samples were seen gradually switching from nearly colorless to reddish-light brown after addition of Ag^+ ions into the filtered cell-free cultures in the dark. The intensity of color has being increased during the time of incubation. After 72 h, the process was stopped and the particles were subjected to further analysis. The reduction of the Ag^+ ions into metal nanoparticles was investigated virtually, so that the color of the three solutions changed into reddish-light brown after 72 h of incubation (Fig. 1A). However, control samples (without silver ions) showed no change in color of the cell filtrates when incubated in the same environmental conditions (Fig. 1B). The appearance of a reddish-light brown color in the reaction suggested the formation of SNP [23]. No precipitation was observed within the solutions even after 72 h of incubation.

UV-visible spectroscopy. Determining UV-visible absorption is a rapid, reliable and available tool for ensuring that colloidal silver particles have been formed. The UV spectroscopy method can be applied for size measurement of SNP based on localized surface plasmon resonance band exhibiting at different wavelength. The light absorption pattern of the cell filtrate was monitored after 72 h in the range of 200-800 nm by using UV-visible spectrophotometer (Fig.

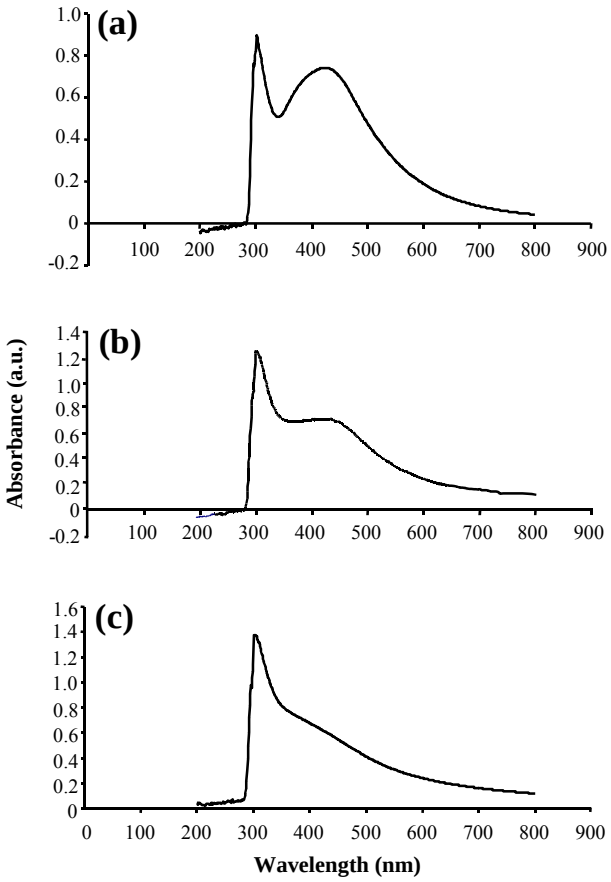

Fig. 2. UV-visible spectrum of liquid mediums containing cell filtrates and silver ion (1 mM) for **(a)** *T. mentagrophytes*, **(b)** *M. canis* and **(c)** *T. rubrum*.

2). As illustrated in Figure 2a, a strong quite narrow peak located between 422 and 425 nm was obtained for *T. mentagrophytes*. The average wavelength at which the peak occurred was around 423.5 nm. Observation of this peak which assigns to a surface plasmon is well-documented for silver metal nanoparticles with sizes much less than 70 nm [24]. Figure 2b indicates a fairly wide peak centering at 441 nm for *M. canis* and Figure 2c shows such a weak spectrum to decipher for *T. rubrum*. However, other tools of determining SNP properties have proved the existence of the particles but with somehow aggression.

Analysis of transmission electron microscopy. TEM analysis was employed to determine the morphology and shape of SNP. The representative TEM image has been indicated in Figure 3. Accordingly for all three testing strains, the majorities of the SNP are relatively uniform in diameter and present in spherical shape. Additionally, TEM images depicted that the particles synthesized by *T. mentagrophytes* are predominantly formed at even less than 50 nm but not for *T. rubrum* and *M. canis*. In other words, the size of particles produced by *M. canis* was more than 50 nm and mostly in the range of 50-70 nm. In case of *T. rubrum*, it was well-demonstrated that the particles were produced in various different sizes, ranging from 50 to more than 100 nm. Moreover, the *T. rubrum* attributed particles were produced in cylindrical rather than spherical shape.

Evaluation of antifungal activity of synthesized SNP. In this study, fluconazole, an antifungal agent which is widely used to treat *Candida*-associated infections, was used as a positive control to compare with SNP. Silver nanoparticles synthesized by *T. mentagrophytes* showed antifungal activity against yeast strain tested and exhibited minimal inhibitory concentration value of 4 µg/mL; however, it was significantly less than fluconazole level. The strain was susceptible dose-dependent against fluconazole, showing minimal inhibitory concentration values of 16 µg/mL toward the fungal strain tested.

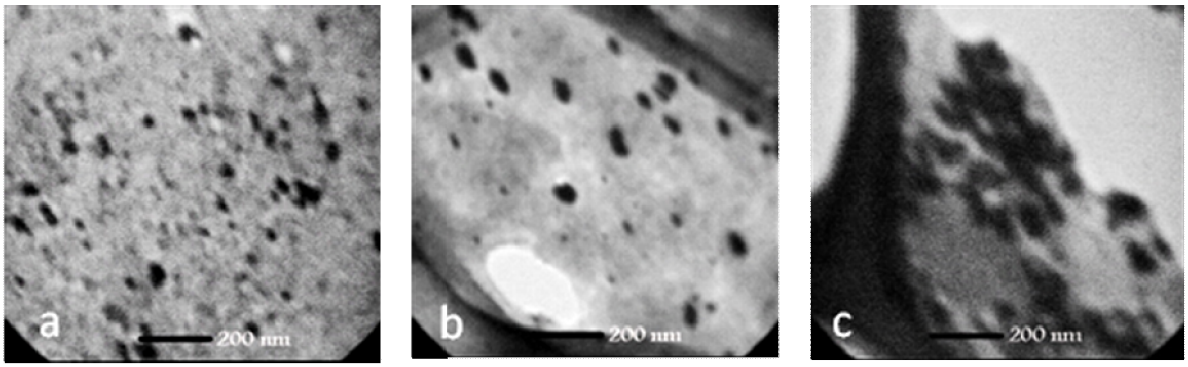

Fig. 3. TEM micrograph of silver particles synthesized by **(a)** *T. mentagrophytes*, **(b)** *M. canis* and **(c)** *T. rubrum* (scale bar: 100 nm).

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DISCUSSION

Myconanotechnology represents a novel field of study in nanotechnology and has currently attracted a great deal of attention. In fact, several scientists have re-explored the fungi including yeasts and filamentous fungi as a biofactory for eco-friendly, cost-effective synthesis of nanoparticles particularly SNP [24, 25]. The advantageous of fungal-mediated biosynthesis of nanoparticles have turned the attention of scientists to the kingdom of fungi. The most notable benefits of applying fungi in nanoscience are their resistance to many harsh conditions as well as secretion of extracellular reductive proteins so that it makes the downstream processes easier [20].

The unusual physicochemical and optoelectronic properties of nanoparticles present them as beneficial candidates for utilization in various fields of natural science including drug and gene delivery [26, 27], biodetection of pathogens [28], probing of DNA structure [29], tumor destruction [30] and tissue engineering [31]. Although living microorganisms are built up cells that are typically 10 μM across, the cell parts are much smaller than this so that the typical size of proteins is just 5 nm which is comparable with the dimensions of artificial nanoparticles [1]. Accordingly, the idea of using nanoparticles as small probes is being arisen. Consequently, it allows researchers to get the inspiration from cellular mechanisms without too much interference [32]. Therefore, innovation of new approaches for cost-effective, non-toxic and eco-friendly synthesis of nanoparticles is preferred nowadays.

The formation of nanoparticles has been reported by many investigators so far, both intracellular and extracellular. For example Ahmad *et al.* [23] reported the ability of the fungus *Fusarium oxysporum* for the synthesis of silver nanoparticles. Sastry *et al.* [26] demonstrated the intracellular synthesis of silver nanoparticles of 2-25 nm within *Verticillium*. Moreover, in 2006, Bhainsa and d'Souza [14] and in 2008, Gade *et al.* [15] reported the ability of soil-born *Aspergillus* to produce extracellulary silver nano-particles. In 2009, Balaji *et al.* [18] used *Cladosporium cladosporioides* for the extracellular synthesis of spherical silver nanoparticles from an aqueous solution of a silver salt. In comparison with other fungi, investigated dermatophytes were able to form bigger nanoparticles except *T. mentagrophytes*.

In the present study, three species of dermatophytes were applied to assess their ability of producing SNP and at last, we achieved notable results which enable us to compare extracellulary the various capacities of different species of dermatophytes in producing SNP.

It was observed that upon addition of the silver ion (1 mM) into the flask containing the cell filtrate, the color of the medium turned into light yellowish-brown (Fig. 1). The intensity of color increased due to excitation of surface plasmon vibrations in the metal nanoparticles. This significant observation indicates that the reduction of the Ag^+ ions takes place extracellulary.

The unique optical properties of metal nanoparticles originate from the collective oscillations of conduction electrons are termed surface plasmon polariton resonances (SPPR) [24]. It is well known that The shape of SPPR spectrum and the wavelength at which the maximum absorbance is occurred is strongly related to the particle size and relative dimensions [33]. Therefore, increasing in particle size makes the SPPR peak move to longer wavelength [33]. Also, the size of SNP has a linear correlation with the peak intensity.

The UV-visible spectrum in Figure 2 shows the SPPR peak of SNP at 422 and 423.5 nm for *T. mentagrophytes* and *M. canis*, respectively. As the spectrums depict, the particles are likely to be less than 70 nm in size and are fairly monodispersed in case of particles synthesized by *T. mentagrophytes*. Unfortunately, no certain peak was observed for *T. rubrum*. Nevertheless, switching of the solution color to yellow as well as evidences of TEM analysis demonstrated that the SNP exist.

In fact, all three strains have successfully synthesized SNP, but *T. mentagrophytes* produced much more desirable particles in aspect of shape and dimensions.

The studies of Duran *et al.* [34] raise the possibility that formation of SNP by *Fusarium oxysporum* is mediated by two distinct mechanisms: One is through nitrate reductase and the other by shuttle quinine process. Similarly, it is also reported that NADH-dependent nitrate reductase is the main enzyme responsible for the reduction of silver ions [35].

Therefore, owing to the fact that the extracellular proteins secreted by the fungi are in charge of the reduction of Ag^+ to Ag^0, it is suggestive to investigate the role of reductase in the fungal filtrate to figure out why some species of dermatophytes produce SNP more efficiently than the other species.

The significant feature of silver is its broad spectrum antimicrobial property. Accordingly, several researchers have studied the antimicrobial activity of SNP against bacterial and fungal strains like *E. coli*, *Staphylococcus aureus* (methicillin-resistant *staphylo-coccus aureus*), *P. aeruginosa, Candida albicans* and HIV virus. Moreover, antimicrobial activity of wound dressings covering with SNP has been successfully examined against several common pathogens [36-39].

Our results for evaluation the antimicrobial properties of synthesized silver nanoparticles were consistent with other researches so that the antifungal properties of the synthesized SNP were accurately proven. In addition to notable anti-*Candida* characteristics, it was well demonstrated that the

inhibitory effect of synthesized SNP was even more effective than the antifungal drug, fluconazole.

In conclusion, extracellular synthesis of SNP is highly beneficial because no extra process is required to separate the particles from the living cells. Dermatophytes have potential for extracellular biosynthesis of SNP. In this investigation, it was observe that species of the same genus of the fungi have the ability to synthesize SNP with different efficiency.

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