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The Silver Nanoparticle (Nano-Ag): a New Model for Antifungal Agents

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1. Introduction

In recent years, a rapid increase in microbes that are resistant to conventional antibiotics has been observed (Goffeau, 2008). Especially, the frequency of infections provoked by opportunistic fungal strains has increased dramatically. Even though the majority of invasive fungal infections are still due to the *Aspergillus* or *Candida* species, the spectrum of fungal pathogens has changed and diversified (Denning, 1991; Ellis et al., 2000; Odds et al., 2003). Azoles that inhibit sterol formation and polyenes that bind to mature membrane sterols have been the mainstays regarding antifungal therapy for several decades (Kullberg & de Pauw, 1999; Sheehan et al., 1999). However, not only the emergence of fluconazole resistance among different pathogenic strains but also the high toxicity of amphotericin B (Alexander & Perfect, 1997; Mukherjee et al., 2003) has prompted research on new antifungal agents (Kontoyiannis et al., 2003).

Bio-nanotechnology has emerged as an integration between biotechnology and nanotechnology for developing biosynthesis and environmental-friendly technology for synthesis of nanomaterials. We specifically regarded nanoparticles as clusters of atoms in the size of 1-100 nm. 'Nano' is a Greek word synonymous to dwarf meaning extremely small. The use of nanoparticles is gaining impetus in the present century as they posses defined chemical, optical and mechanical properties. Among them, the metallic nanoparticles are most promising as they contain remarkable antibacterial properties due to their large surface area to volume ratio, which is of interest to researchers due to the growing microbial resistance against metal ions, antibiotics, and the development of resistant strains (Rai et al., 2009; Gong et al., 2007).

Different types of nanomaterials like copper, zinc, titanium (Schabes-Retchkiman et al., 2006), magnesium, gold (Gu et al., 2003), alginate (Ahmad et al., 2005) and silver have been developed but silver nanoparticles (Nano-Ag) have proved to be most effective as they exhibit potent antimicrobial efficacy against bacteria, viruses and eukaryotic micro-organisms. However, Nano-Ag used as a disinfectant drug also has some risks as the exposure to silver can cause argyrosis or argyria; it can be toxic to mammalian cells (Gong et al., 2007).

The current investigation supports the theory that the use of silver ions or metallic silver as well as Nano-Ag can be exploited in medicine for burn treatment, dental materials, coating stainless steel materials, textile fabrics, water treatment, sunscreen lotions, etc. and posses low toxicity to human cells, high thermal stability and low volatility (Duran et al., 2007).
Many studies have shown the biological effects of Nano-Ag, however, its effects against fungal pathogens have not yet been fully studied. In this study, therefore, the antifungal properties and mechanism of actions of Nano-Ag against human pathogenic fungal strains were investigated. Furthermore, the therapeutic potential of Nano-Ag for treating fungal diseases in humans was suggested.

2. Materials and methods

2.1. Materials

Amphotericin B, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), trehalase and RNase A were purchased from the Sigma Chemical Co.. Stock solutions of amphotericin B were prepared in dimethyl sulfoxide (DMSO), and stored at -20 ˚C. For all the experiments, a final concentration of 2% DMSO was used as the solvent carrier.

2.2. Preparation of Nano-Ag

One hundred grams of solid silver were dissolved in 100 ml of 100% nitric acid at 90 ˚C, and then 1 l of distilled water was added. By adding sodium chloride to the silver solution, the Ag ions were precipitated and then clustered together to form monodispersed nanoparticles in the aqueous medium. The sizes and morphology of Nano-Ag were examined by using a transmission electron microscope (TEM) (H-7600, HITACHI, LTD). The result showed that the Nano-Ag was in a spherical form and had an average size of 3 nm (Fig. 1).

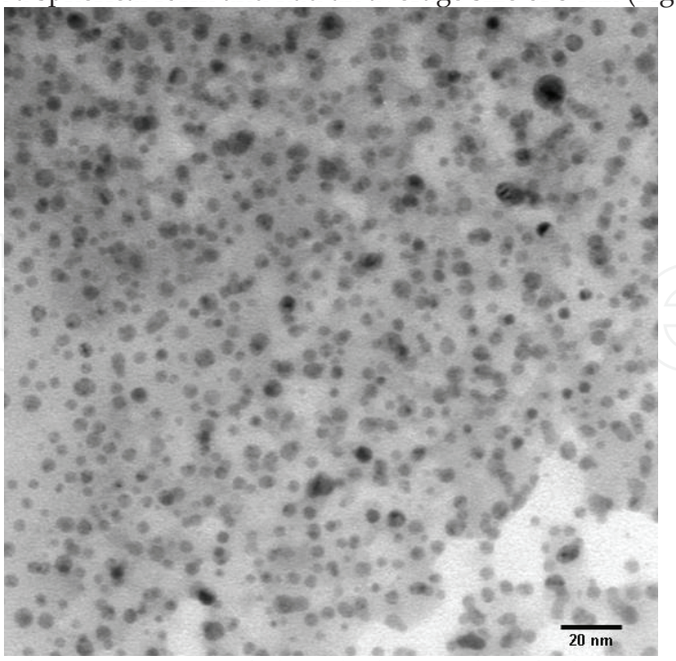


Fig. 1. Transmission electron micrograph (TEM) of Nano-Ag. The bar corresponds to 20 nm.

As the final concentration of colloidal silver was 60,000 ppm, this solution was diluted, and then samples of different concentrations were used to investigate the antifungal effects of Nano-Ag.

2.3. Fungal strains and culture conditions

A total of 44 strains of 2 fungal species were used in this study. *Candida albicans* (ATCC 90028), *Candida glabrata* (ATCC 90030), *Candida parapsilosis* (ATCC 22019), and *Candida krusei* (ATCC 6258) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Clinical isolates of *Candida* spp. were obtained from the Department of Laboratory Medicine, Chonnam National University Medical School (Gwangju, Korea), and clinical isolates of *Trichophyton mentagrophytes* were obtained from the Institute of Medical Mycology, Catholic Skin Clinic (Daegu, Korea). *Candida* spp. and *Trichophyton mentagrophytes* were cultures in a Sabouraud dextrose agar (SDA) and a potato dextrose agar (PDA) at 35 ˚C, respectively.

2.4. Antifungal susceptibility testing

The minimum inhibitory concentration (MIC) for *Candida* spp. and *T. mentagrophytes* was determined by a broth microdilution method based on the National Committee for Clinical Laboratory Standards (NCCLS; now renamed as Clinical and Laboratory Standards Institute, CLSI, 2000) method outlined in documents M-27A and M-38P, respectively. An RPMI 1640 medium buffered to pH 7.0 with 3-(*N*-morpholino) propanesulfonic acid (MOPS) was used as the culture medium, and the inoculum size of *Candida* spp. was 0.5×10^3 to 2.5×10^3 cells/ml, and that of *T. mentagrophytes* was 0.4×10^4 to 5×10^4 cells/ml. The microdilution plates inoculated with fungi were incubated at 35 ˚C, and the turbidity of the growth control wells was observed every 24 hrs. The 80% inhibitory concentration (IC_{80}) was defined as the lowest concentration that inhibited 80% of the growth as determined by a comparison with the growth in the control wells. The growth was assayed with a microplate reader (Bio-Tek Instruments, Winooski, VT, USA) by monitoring absorption at 405 nm. In the current study, amphotericin B and fluconazole were used as a positive control toward fungi; amphotericin B is a fungicidal agent widely used in treating serious systemic infections (Hartsel & Bolard, 1996), and fluconazole is used in the treatment of superficial skin infections caused by dermatophytes and *Candida* species (Amichai & Grunwald, 1998).

2.5. The effect on the dimorphic transition of *C. albicans*

C. albicans cells were maintained by periodic subculturing in a liquid yeast extract/peptone/dextrose (YPD) medium. Cultures of yeast cells (blastoconidia) were maintained in a liquid YPD medium at 37 ˚C. To induce mycelial formation, cultures were directly supplemented with 20% of fetal bovine serum (FBS). The dimorphic transition in *C. albicans* was investigated from cultures containing 2 mg/ml of Nano-Ag (at the IC_{80}), which were incubated for 48 hrs at 37 ˚C (Jung et al., 2007; Sung et al., 2007). The dimorphic transition to mycelial forms was detected by phase contrast light microscopy (NIKON, ECLIPSE TE300, Japan).

2.6. Hemolytic activity assay

The hemolytic effect of Nano-Ag was examined by measuring the release of hemoglobin from a 4% suspension of fresh human red blood cells (hRBCs). The hRBCs were washed with phosphate-buffered saline (PBS: 35 mM phosphate buffer/150 mM NaCl, pH 7.4). One–hundred microliters of hRBC suspension was added to the 96-well microtiter plates, and then 100 µl of the compound solution in PBS was mixed into each well. After incubating the mixtures for 1 hr at 37 ˚C, the mixtures were centrifuged at 1,500 rpm for 10 min, and the aliquots were transferred to new 96-well microtiter plates. The absorbance of the aliquots was measured at 414 nm by using a microtiter ELISA Reader. Hemolytic rates of 0 and 100% were determined in PBS and 0.1% Triton X-100, respectively (Park et al., 2003). The percentage of hemolysis was calculated by employing the following equation:

$$\text{Percentage hemolysis} = [(Abs_{414\,nm} \text{ in the compound solution} - Abs_{414\,nm} \text{ in PBS})/(Abs_{414\,nm} \text{ in 0.1\% Triton X-100} - Abs_{414\,nm} \text{ in PBS})] \times 100.$$

2.7. Flow cytometric analysis for plasma membrane potential

For analysis of the membrane integrity after the treatment of Nano-Ag, log-phased cells of *C. albicans* (1 x 10^8 cells), cultured in a YPD medium, were harvested and resuspended with 1 ml of a fresh YPD medium, containing 30 µg/ml of Nano-Ag (at 15 times the MIC) or 10 µM of CCCP, used as a positive control. After incubation for 3 hrs, the cells were washed three times with PBS. To detect any depolarization of the cell membrane, 1 ml of PBS, containing 50 µg of bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$], was added and the samples were incubated for 1 hr at 4°C in the dark (Liao et al., 1999). Flow cytometric analysis was performed using a FACSCalibur flow cytometer.

2.8. Measurement of plasma membrane fluorescence intensity

The fluorescence intensity from exponential *C. albicans* cells labeled by 1,6-diphenyl-1,3,5-hexatriene (DPH) was used to monitor changes in membrane dynamics. The cells (1 x 10^8 cells in a YPD medium), containing 20, 40, 60 and 80 µg/ml of Nano-Ag or amphotericin B, were incubated at a physiological temperature of 28°C on a rotary shaker at 140 rpm for 2 hrs. The control cells were incubated without a compound. The cells were fixed with formaldehyde (0.37%, v/v) for 45 min and they were collected by centrifugation at 3,000 rpm, then washed several times with PBS buffer (pH 7.4), and the pellets were frozen in liquid nitrogen. For DPH labeling, the pellets were resuspended in PBS buffer and incubated at 28°C for 45 min in the presence of 0.6 mM of DPH, followed by several washings in PBS buffer. The steady-state fluorescence intensity was measured by using a SHIMADZU RF-5301PC spectrofluorophotometer at 350 nm excitation and 425 nm emission wavelengths (Fernandes et al., 2000). The results represent the average of the triplicate measurements from three independent assays.

2.9. Determining released glucose and trehalose

Fungal strains were grown at 28°C in a YPD medium. *C. albicans* cells were washed three times with PBS, and then 1 ml of the *C. albicans* cell suspension (1 x 10^8 cells), containing 20 µg/ml of Nano-Ag (at 20 times the MIC), was incubated for 2 hrs at 28°C in PBS. The negative control was incubated without Nano-Ag, and a positive control was incubated

with 100 μg/ml of amphotericin B (at 20 times the MIC). The fungal cells were settled by centrifugation (12,000 rpm for 20 min). The pellets were dried to calculate their dry weight and supernatants were transferred to a new tube. Released glucose and trehalose-containing supernatants were added to 0.05 units of trehalase. After 1 hr of enzymatic reaction at 37°C, the reaction suspension was mixed with water and 16% DNS reagent (3,5-dinitrosalicylic acid 1%, NaOH 2%, sodium potassium tartrate 20%) was added. For the reaction of glucose with the DNS reagent, the mixture was boiled for 5 min and cooled. Color formations were measured at 525 nm. The results represent the average of the measurements conducted in triplicate of three independent assays.

2.10. Transmission electron microscopy (TEM)

Log-phased cells of *C. albicans* (1 x 10^8 cells) cultured in a YPD medium, were harvested and incubated in the presence of several different amounts of Nano-Ag for 24 hrs at 28°C. TEM was used as a complementary technique to examine sections of the treated cells, using standard procedures for fixing and embedding sensitive biological samples, which are described elsewhere (Osumi, 1998; Mares et al., 1998).

2.11. Flow cytometric analysis for a fungal cell cycle

Log-phased cells of *C. albicans* (1 x 10^8 cells) cultured in a YPD medium, were harvested and treated with 40 μg/ml of Nano-Ag (at 20 times the MIC). After incubation for 8 hrs, the cells were washed with PBS and fixed with 70% ethanol overnight at 4°C. The cells were treated with 200 μg/ml of RNase A and the mixture was left to react for 2 hrs at 37°C. For DNA staining, 50 μg/ml of propidium iodide (PI) were added and incubated for 1 hr at 4°C in the dark (Green et al., 1999). Flow cytometric analysis was performed by a FACSCalibur flow cytometer. The values represent the average of the measurements conducted in triplicate of three independent assays.

3. Results and discussion

3.1. Antifungal activity of Nano-Ag

In the past, silver has been in use for the treatment of burns or chronic wounds. In the 1940s, however, after penicillin was introduced, the use of silver for the treatment of bacterial infections decreased (Hugo & Russell, 1982; Demling & DeSanti, 2001; Chopra, 2007).

Silver again came in picture in the 1960s when Moyer introduced the use of 0.5% silver nitrate for the treatment of burns. He proposed that this solution does not interfere with epidermal proliferation and possess antibacterial properties against *Staphylococcus aureus, Pseudomonas aeruginosa and Escherichia coli* (Moyer et al., 1965; Bellinger & Conway, 1970). In 1968, silver nitrate was combined with sulfonamide to form silver sulfadazine cream, which served as a broad-spectrum antibacterial agent and was used for the treatment of burns. Silver sulfadazine is effective against various bacterial strains like *E. coli*, *S. aureus*, *Klebsiella* sp., and *Pseudomonas* sp. (Rai et al., 2009). It also possesses some antifungal and antiviral activities (Fox & Modak, 1974). Recently, due to the emergence of antiobiotic-resistant bacteria and limitations of the use of antibiotics clinicians have returned to using silver wound dressings, containing varying level of silver (Gemmell et al., 2006; Chopra, 2007). For

these reasons, the antifungal activity and its mechanism of silver, Nano-Ag specifically, was investigated.

Nano-Ag, in an IC_{80} range of 1-25 μg/ml, showed significant antifungal activity against *T. mentagrophytes* and *Candida* species. Toward all fungal strains, Nano-Ag exhibited similar activity with amphotericin B, showing IC_{80} values of 1-5 μg/ml, but more potent activity than fluconazole, showing IC_{80} values of 10-30 μg/ml. However, this compound exhibited less potent activity than amphotericin B, showing IC_{80} values of 2-4 μg/ml for *C. parapsilosis* and *C. krusei* (Table 1).

Fungal strains (no. of strains)	IC_{80} (μg/ml)		
	Nano-Ag	Amphotericin B	Fluconazole
C. albicans (4)	2-4	5	10-16
C. tropicalis (2)	7	2-4	13
C. glabrata (4)	1-7	2	10-16
C. parapsilosis (3)	4-25	2	13
C. krusei (1)	13	4	13
T. mentagrophytes (30)	1-4	1-2	20-30

Table 1. Antifungal activity of Nano-Ag.

In order to elucidate the antifungal activity of Nano-Ag, the dimorphic transition of *C. albicans*, induced by Nano-Ag, was investigated. The dimorphic transition of *C. albicans* from yeast form to mycelial form is responsible for pathogenicity, with mycelial shapes being predominantly found during the invasion of host tissue. A mycelial form can be induced by temperature, pH, and serum (McLain et al., 2000). As shown in Fig. 2, the serum-induced mycelia were significantly inhibited from extending and forming in the presence of Nano-Ag (Fig. 2C), but the mycelia formed was normal in the absence of Nano-Ag (Fig. 2B).

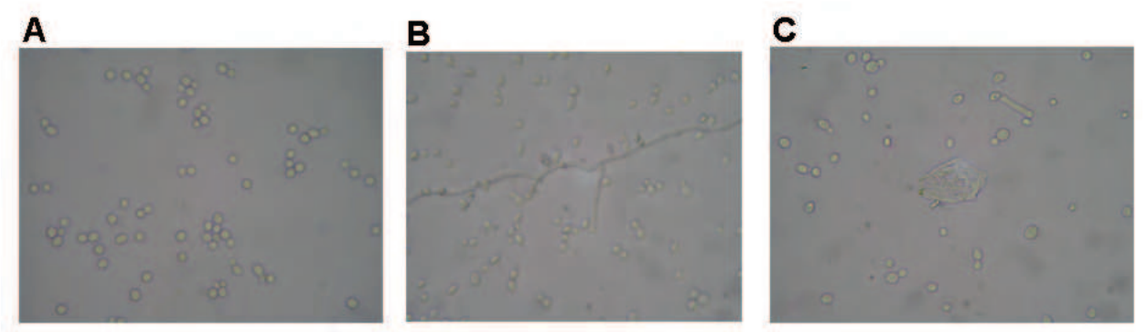


Fig. 2. The effect of Nano-Ag on the dimorphic transition in *C. albicans*. (A) Yeast control without 20% FBS and Nano-Ag, (B) Cells treated with only 20% FBS, (C) Cells treated with 2 μg/ml of Nano-Ag.

These results confirmed the antifungal activity of Nano-Ag and suggested that Nano-Ag can be a potential compound in the treatment of fungal infectious diseases.

3.2. Hemolytic activity of Nano-Ag against human erythrocytes

Many antimicrobial agents are limited regarding clinical applications, as they can induce cytolysis of human cells. The hemolytic activity of Nano-Ag was investigated as an indicator

of its cytotoxicity to mammalian cells. The hemolytic activity was evaluated by the percentage of hemolysis in the concentration range of 1.25-10 μg/ml. The result showed that Nano-Ag caused 6% lysis of erythrocytes at a concentration of 10 μg/ml, whereas amphotericin B induced 10% lysis at the same level (Table 2).

Compound	% Hemolysis (μg/ml)			
	10	5	2.5	1.25
Nano-Ag	6	0	0	0
Amphotericin B	10	0	0	0

Table 2. Hemolytic activity of Nano-Ag against human erythrocytes.

This result suggested that Nano-Ag could be applied to therapeutic agents regarding human fungal diseases with low cytotoxicity.

3.3. Changes of plasma membrane potential induced by Nano-Ag

To assess whether Nano-Ag can affect the function of a fungal plasma membrane, the dissipation of the fungal plasma membrane potential was investigated. *C. albicans* cells were cultured in the presence of Nano-Ag or CCCP used as a positive control, and the amounts of accumulated $DiBAC_4(3)$ in the cells were measured *via* flow cytometry by staining with $DiBAC_4(3)$. CCCP is an H^+ ionophore which dissipates the H^+ gradient and thus uncouples electron transport from ATP synthesis (Ghoul et al., 1989). $DiBAC_4(3)$ has a high voltage sensitivity and it enters depolarized cells, where it binds to lipid-rich intracellular components (Liao et al., 1999). Therefore, the fluorescence of $DiBAC_4(3)$ increases upon membrane depolarization. The addition of Nano-Ag to *C. albicans* cells caused an increase in fluorescence intensity, similar to the increase observed in the presence of CCCP, which is indicative of membrane depolarization (Fig. 3). This result indicated that Nano-Ag affected yeast cells by attacking their membranes, thus disrupting membrane potential.

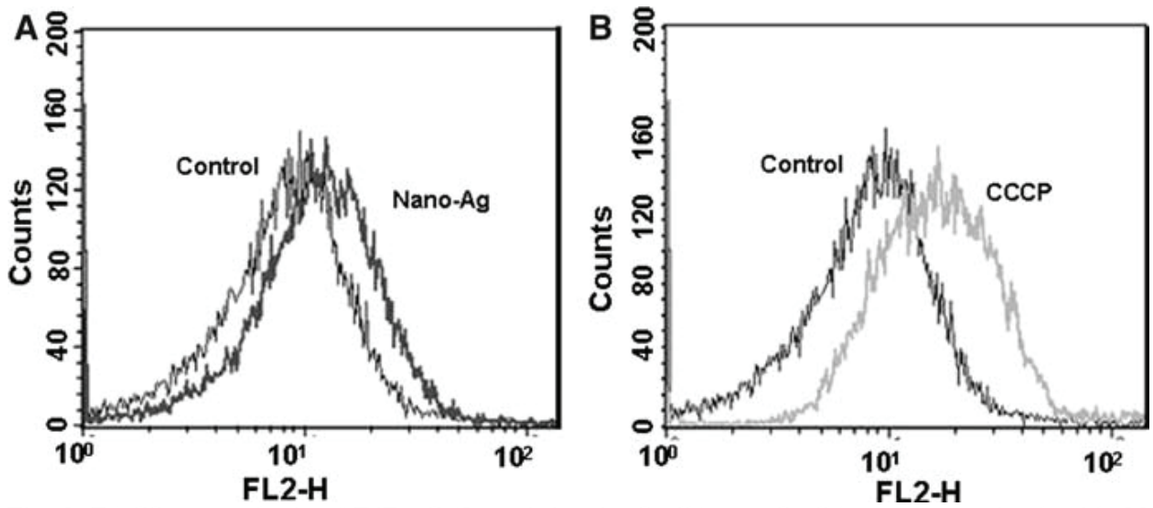


Fig. 3. FACScan analysis of $DiBAC_4(3)$ staining in *C. albicans*. *C. albicans* were mixed with 30 μg/ml of Nano-Ag and 10 μM of CCCP and incubated at 28 ˚C for 3 hrs under constant shaking. This figure shows the fluorescence intensity of stained *C. albicans* treated with the

compounds. FL2-H indicates the log fluorescent intensity of $DiBAC_4(3)$, and y-axis indicates cell numbers (events).

3.4. Changes in the plasma membrane dynamics of fungal cells induced by Nano-Ag

The effect of Nano-Ag on the fungal plasma membrane was further investigated by using DPH as a membrane probe. If the antifungal activities exerted by Nano-Ag on *C. albicans* are at the level of the cell plasma membrane, DPH, which can interact with an acyl group of the plasma membrane lipid bilayer, can not be inserted into the membrane. As shown in Fig. 4, the plasma membrane DPH fluorescence anisotropy was significantly decreased by increasing the concentrations of Nano-Ag or amphotericin B. This is consistent with the disruption of the plasma membrane by Nano-Ag as well as by a positive control, amphotericin B.

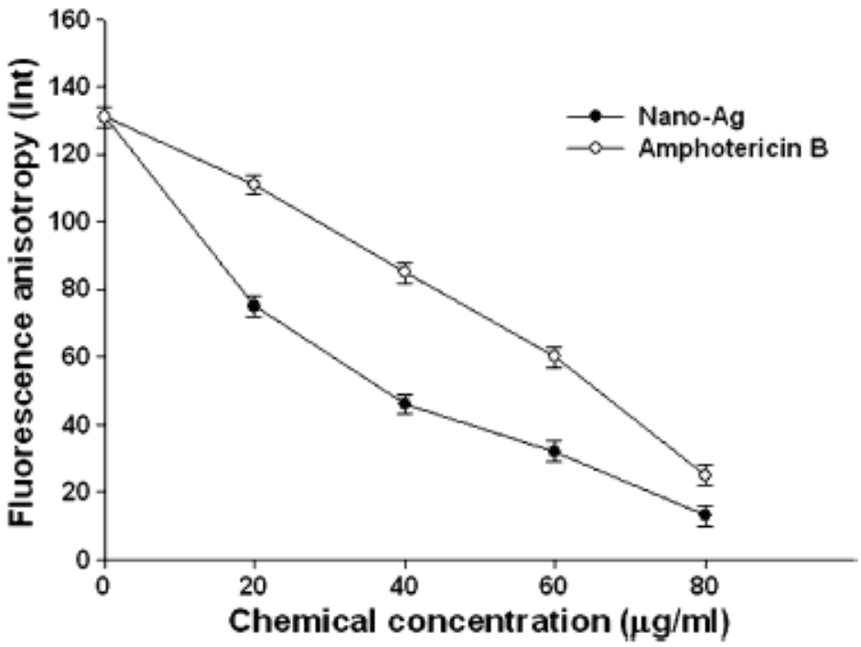


Fig. 4. DPH fluorescence anisotropy after the addition of Nano-Ag or amphotericin B. The error bars represent the standard deviation (SD) values for three independent experiments, performed in triplicate.

3.5. Intracellular glucose and trehalose release induced by Nano-Ag

The ability of Nano-Ag to disturb the integrity of the plasma membrane of fungal cells was also assessed by measuring the glucose and trehalose released in cell suspensions which were exposed to this compound. Trehalose can protect proteins and biological membranes from inactivation or denaturation caused by a variety of stress conditions, including desiccation, dehydration, heat, cold, oxidation, and toxic agents in yeast (Alvarez-Peral et al., 2002; Elbein et al., 2003). The result showed that Nano-Ag or amphotericin B-treated cells both accumulated more intracellular glucose and trehalose than the compound-untreated cells. In addition, these cells also increased extracellular glucose and trehalose than the

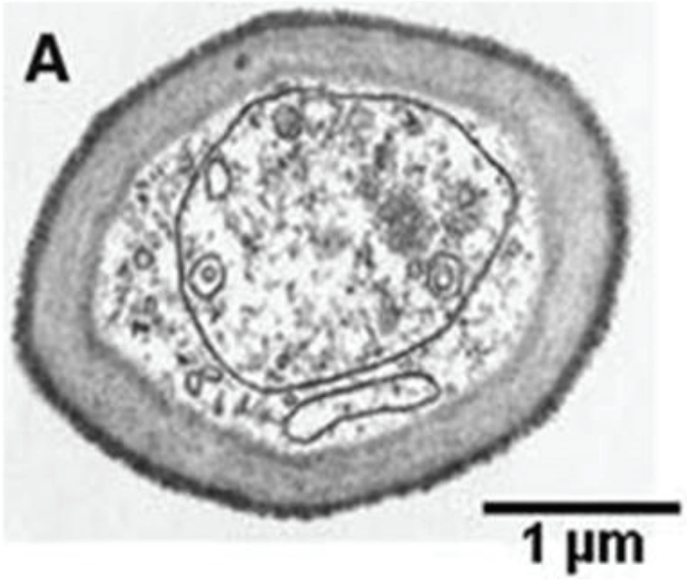
compound-untreated cells. The extracellular glucose and trehalose, induced by amphotericin B, were measured as being 27.4 μg per fungal dry weight of 1 mg. The extracellular glucose and trehalose amounts, however, induced by Nano-Ag were measured as being 30.3 μg per fungal dry weight of 1 mg. This rate was significantly higher than that induced in the compound-untreated cells (Table 3). The result suggests that it may be one of several intracellular components released during membrane disruption by Nano-Ag. As for the mechanism by which Nano-Ag breaks down the membrane permeability barrier, it is possible that Nano-Ag perturbs the membrane lipid bilayers, causing the leakage of ions and other materials as well as forming pores and dissipating the electrical potential of the membrane.

	Amounts of trehalose and glucose concentrations (μg/mg)	
	Intracellular glucose and trehalose	Released glucose and trehalose
Control	7.2	6.8
Nano-Ag	16.1	30.3
Amphotericin B	20.5	27.4

Table 3. The concentrations of trehalose and glucose from *C. albicans* by Nano-Ag and amphotericin B.

3.6. Transmission electron microscopic (TEM) analysis

TEM was used to evaluate the ability of Nano-Ag to disrupt the fungal envelope structure. The results showed that the Nano-Ag-treated fungal cells showed significant damage, which was characterized by the formation of a "pit" in their cell walls and pores in their plasma membrane (Fig. 5).



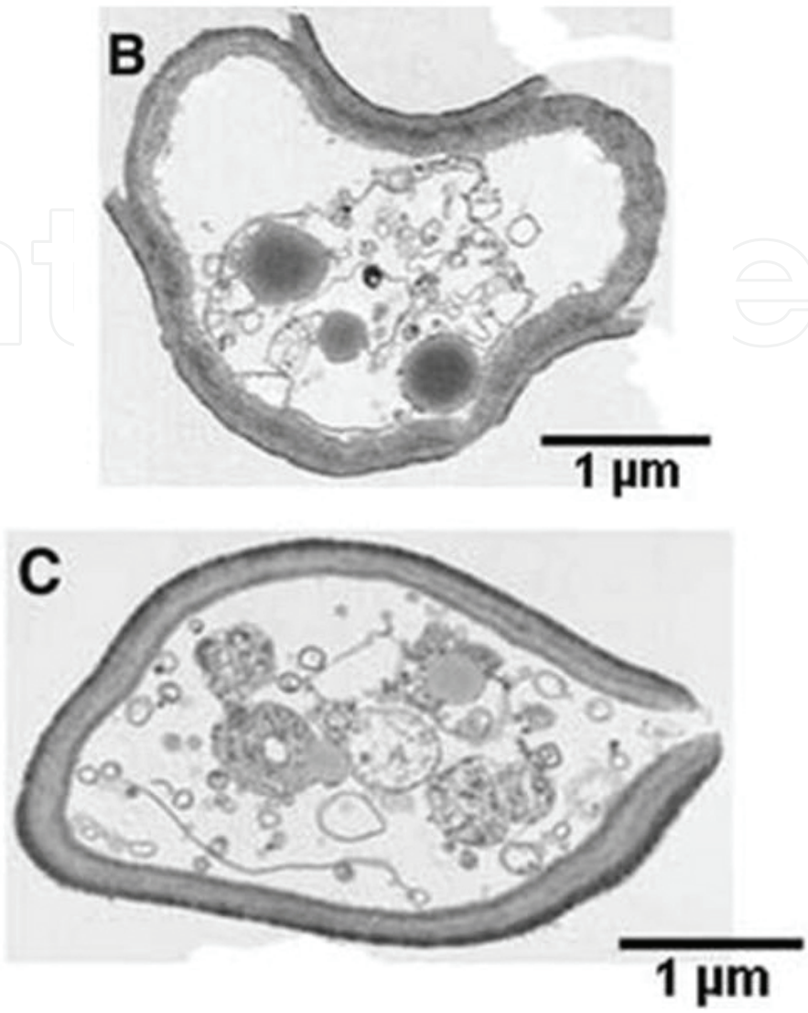


Fig. 5. Transmission electron micrograph (TEM) of *C. albicans* cells. *C. albicans* were incubated in the presence of different amounts of Nano-Ag for 24 hrs at 28 ˚C. The bar marker represents 1 μm. (A) Control, (B) Cells treated with 170 μg/ml of Nano-Ag, (C) Cells treated with 400 μg/ml of Nano-Ag.

3.7. The arrest of fungal cell cycle induced by Nano-Ag

In order to elucidate the physiological changes of the fungal cells induced by Nano-Ag, a flow cytometric analysis of the cell cycle was performed. The cells were cultured in the presence or absence of Nano-Ag and their DNA content was determined *via* flow cytometry by staining with propidium iodide (PI). PI is a DNA-staining dye that intercalates between the bases of DNA or RNA molecules (Tas & Westerneng, 1981). As shown in Fig. 6, the percentage of cells in the G_2/M phase increased by 15%, while that in the G_1 phase significantly decreased by about 20% in the presence of Nano-Ag. This data suggests that Nano-Ag inhibited some cellular processes which are involved in normal bud growth. Endo et al. have reported that the inhibition of bud growth correlates with membrane damage

(Endo et al., 1997). Therefore, this report suggests that Nano-Ag inhibits the normal budding process, probably through the destruction of membrane integrity.

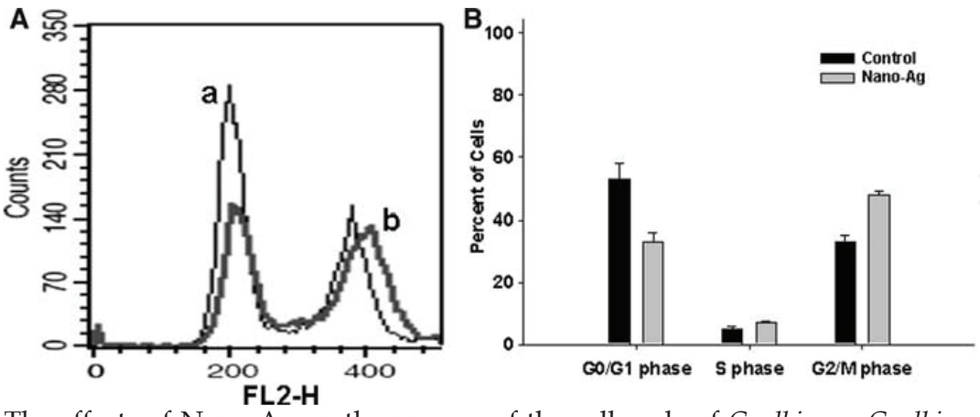


Fig. 6. The effects of Nano-Ag on the process of the cell cycle of *C. albicans*. *C. albicans* were incubated in the presence of Nano-Ag for 8 hrs at 28 ˚C. FL2-H indicates the fluorescent intensity of PI, and y-axis indicates cell numbers (events). (A) FACS diagram of cell cycle; (a) Control without Nano-Ag, (b) Cells treated with 40 μg/ml Nano-Ag, (B) A histogram indicating the percentage of each cell cycle progress.

4. Conclusion

Many studies have shown the antimicrobial effects of Nano-Ag (Klasen, 2000; Russell & Hugo, 1994; Silver, 2003), but the effects of Nano-Ag against fungal pathogens of the skin, including clinical isolates of *T. mentagrophytes* and *Candida* species are mostly unknown. The primary significance of this study is the observation that Nano-Ag can inhibit the growth of dermatophytes, which cause superficial fungal infections (Kim et al., 2008). Nano-Ag also exhibited potent antifungal effects, probably through destruction of membrane integrity (Kim et al., 2009). To the author's knowledge, this is the first study to apply Nano-Ag successfully to dermatophytes and pathogenic fungal strains. Secondly, the fact that preparation method of Nano-Ag described here is cost-effective is also of importance. Therefore, it can be expected that Nano-Ag may have potential as an anti-infective agent for human fungal diseases.

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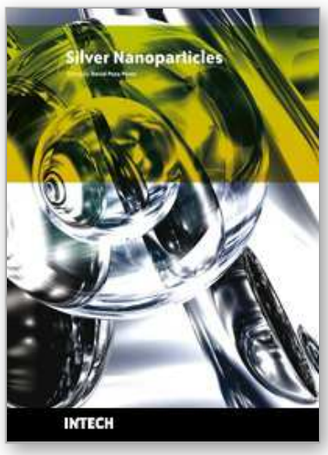

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