BRIEF REPORT

Demonstration of Antibiofilm and Antifungal Efficacy of Chitosan against Candidal Biofilms, Using an In Vivo Central Venous Catheter Model

Luis R. Martinez,[1,2,6,a] Mircea Radu Mihu,[1,2,a] Moses Tar,[3] Radames J. B. Cordero,[1,2] George Han,[4] Adam J. Friedman,[4,5] Joel M. Friedman,[4,5] and Joshua D. Nosanchuk[1,2]

[1]Division of Infectious Diseases, Department of Medicine, and Departments of [2]Microbiology and Immunology, [3]Urology, [4]Physiology and Biophysics, Albert Einstein College of Medicine, and [5]Division of Dermatology, Department of Medicine, Montefiore Medical Center, and [6]Department of Biology and Medical Laboratory Technology, Bronx Community College, Bronx, New York

Candida **species are a major cause of catheter infections. Using a central venous catheter *Candida albicans* biofilm model, we demonstrated that chitosan, a polymer isolated from crustacean exoskeletons, inhibits candidal biofilm formation in vivo. Furthermore, chitosan statistically significantly decreased both the metabolic activity of the biofilms and the cell viability of *C. albicans* and *Candida parapsilosis* biofilms in vitro. In addition, confocal and scanning electron microscopic examination demonstrated that chitosan penetrates candidal biofilms and damages fungal cells. Importantly, the concentrations of chitosan that were used to evaluate fungal biofilm susceptibility were not toxic to human endothelial cells. Chitosan should be considered for the prevention or treatment of fungal biofilms on central venous catheters and perhaps other medical devices.**

Among vascular catheter–related infections, those due to *Candida* species are associated with the highest mortality rate [1]. Successful therapy for these foreign-body infections is therapeutically challenging [2]. Current guidelines for the treatment of catheter-associated candidemia advocate catheter removal [3]. In contrast to removal of peripheral intravenous catheters, removal of larger central venous catheters (CVCs) is not always feasible, and replacement is expensive and associated with a procedural risk for the patient.

Biofilms, in which microorganisms encased in a self-produced extracellular matrix are protected from antimicrobial agents and host cells, represent unique niches for microbial growth. Microorganisms disseminate from the self-contained environment of biofilms, which leads to persistent infections. Fungi can form biofilms on biomaterials that are commonly used in clinical practice (eg, CVCs, dentures, and heart valves) and are associated with establishment and progression of disease [4, 5].

Chitosan, a hydrophilic biopolymer that is industrially obtained by means of *N*-deacetylation of crustacean chitin, exhibits antimicrobial activity against a wide variety of microorganisms [6]. Recently, surfaces coated with chitosan were shown to resist biofilm formation by bacteria and fungi in vitro [7]. In the present work, we used a CVC *Candida albicans* biofilm model [8] to study the in vivo efficacy of chitosan for biofilm prevention. Furthermore, we determined the susceptibility of mature *C. albicans* and *Candida parapsilosis* biofilms to chitosan in vitro. Our results show that this natural compound potentially can be developed as a therapeutic agent for the prevention and treatment of catheter-associated *Candida* species infections and perhaps additional fungal biofilm-based diseases.

Methods. *C. albicans* SC5314 was obtained from Mahmoud Ghannoum (Cleveland, OH). *C. parapsilosis* GA-1, a clinical isolate, was obtained from Karsten Neuber (Hamburg, Germany). Both strains were grown in yeast extract, peptone, and dextrose (YPD) broth (Difco Laboratories) containing 10 g/L yeast extract, 10 g/L peptone, and 20 g/L glucose for 24 h at 30°C with shaking at 150 rpm (to early stationary phase).

A *C. albicans* CVC biofilm model was used for in vivo experiments, as described elsewhere [8]. Chitosan-treated CVCs were generated by incubating polyethylene catheters (PE 100 [inner diameter, 0.76 mm; outer diameter, 1.52 mm]; Intramedic catalog no. 427430; Becton Dickinson) with 5 mg/mL chitosan for 1 h at room temperature. Briefly, female Sprague-Dawley rats weighing 400 g (Harlan Sprague-Dawley) were anesthetized, and the right external jugular was exposed. A longitudinal incision was made in the vein wall, and a sterile, heparinized (100 U/mL) control or chitosan-treated catheter was inserted at a site above the right atrium (~2 cm) and

Received 28 April 2009; accepted 22 July 2009; electronically published 23 March 2010.
Potential conflicts of interest: none reported.
Financial support: National Institutes of Health (grant T32AI007506-11A1 to L.R.M. and grant AI056070-01A2 to J.D.N.); Department of Defense (grant DAMD17-03-1-0127 to J.M.F. and A.J.F.); Orlando Dermatology and Aesthetic Conference (grant to A.J.F.).
[a] L.R.M. and M.R.M. contributed equally to this work.
Reprints or correspondence: Luis R. Martinez, Albert Einstein College of Medicine, 1300 Morris Park Ave, Bronx, NY 10461 (lmartine@aecom.yu.edu).

The Journal of Infectious Diseases 2010; 201(9):1436–1440

0022-1899/2010/20109-0021$15.00
DOI: 10.1086/651558

secured with 3–0 silk ties. The proximal end of the catheter was tunneled subcutaneously and secured on the subscapular skin by means of a button secured with a 2–0 Ti-Cron suture. The wound was closed with staples (Ethicon Endo-Surgery). After surgery, an inoculum of 1×10^6 cells/mL *C. albicans* washed and suspended in phosphate-buffered saline (PBS) was instilled in the catheter lumen. Catheters were collected 24 h after infection.

To assess biofilm formation, scanning electron microscopy was used to examine the catheters. The catheters were transected lengthwise, fixed overnight (4% formaldehyde and 1% glutaraldehyde in PBS), washed for 5 min in PBS, and placed in 1% osmium tetroxide for 30 min. After a series of alcohol washes, the samples were critical-point dried, mounted, gold coated, and viewed in a JEOL JSM-6400 scanning electron microscope in high-vacuum mode at 10 kV.

To explore the effects of chitosan on *Candida* species, several in vitro studies were performed. Fungal cells grown in YPD medium were collected, washed twice with PBS, and suspended at 1×10^7 cells/mL in a defined minimal medium (pH, 5.5; 20 mg/mL thiamine, 30 mmol/L glucose, 26 mmol/L glycine, 20 mmol/L magnesium sulfate heptahydrate, and 58.8 mmol/L potassium dihydrogen phosphate). One hundred microliters of the suspension was added to individual wells of 96-well polystyrene plates and incubated without shaking at 37°C. Biofilms were allowed to form for 48 h, and the wells were washed 3 times with 0.05% Tween 20 in Tris-buffered saline with use of a plate washer (Skan Washer 400; Molecular Devices). Fungal cells that remained attached to the plastic surface were considered to be in biofilms, as documented by microscopic examination (data not shown).

To evaluate the susceptibilities of biofilms to chitosan, 200 μL of PBS containing chitosan (0, 0.625, 1.25, 2.5, and 5 mg/mL) was added to each well. *Candida* species biofilms and chitosan were mixed and incubated without shaking at 37°C for 0.5 or 1 h. After incubation, biofilm metabolic activity and fungal survival were determined. A semiquantitative measurement of biofilm formation was obtained by use of the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H–tetrazolium hydroxide (XTT) reduction assay [9]. The colorimetric change was measured at 492 nm (Multiskan; Labsystems). To determine the number of colony-forming units, biofilms were scraped from the bottom of the wells with a sterile 200-μL micropipette tip to dissociate yeast cells. A 100-μL volume was aspirated from the wells, transferred to a microcentrifuge tube with 900 μL of PBS, and vortexed gently for 3 min. The samples were serially diluted onto YPD agar plates and incubated at 37°C, and colony-forming units were enumerated.

For scanning electron microscopic examination of biofilm in vitro, candidal biofilms were grown on glass coverslips in 6-well microtiter plates with minimal medium at 37°C for 48 h, followed by incubation with PBS or 1.25 mg/mL chitosan at 37°C for 1 h. Coverslips were then washed 3 times with PBS and processed for scanning electron microscopic examination as described above.

For confocal microscopic examination, *Candida* species biofilms subsequently incubated with or without 1.25 mg/mL chitosan at 37°C for 1 h were incubated for 45 min at 37°C in 75 μL of PBS containing the fluorescent stains FUN-1 (10 μmol/L) and concanavalin A-alexa fluor 488 conjugate (ConA; 25 μmol/L; Molecular Probes). Confocal microscopic examination of the biofilms was performed with an Axiovert200M (objective, ×40; numerical apertures, 0.6). Depth measurements across the width of the device were taken at regular intervals. To determine structure, a series of horizontal (*x-y*) optical sections with a thickness of 1.175 μm were taken throughout the full length of the biofilm. Confocal images of green (ConA) and red (FUN-1) fluorescence were recorded simultaneously using a multichannel mode. *Z*-stack images and measurements were corrected using Axio Vision software (version 4.4; Carl Zeiss MicroImaging) in the deconvolution mode.

The ζ potential was calculated with a ZetaPlus analyzer (Brookhaven Instruments) to assess the effect of chitosan on fungal cell membrane during biofilm formation. The ζ potential is a measurement (in millivolts) of the electric potential gradient that develops across the interface between a boundary liquid in contact with a solid and the mobile diffuse layer in the body of the liquid. Yeast cells were incubated with or without chitosan for 1 h at 37°C, extensively washed in PBS, and diluted in distilled water, and the ζ potential was measured with the ZetaPlus analyzer.

Statistical analyses were performed with Prism software (version 5.0; GraphPad). Analysis of variance, adjusted by use of the Bonferroni correction, was used to calculate *P* values. Results for which $P < .05$ were considered statistically significant.

Results. We used a previously characterized CVC model to mimic device-associated infection to determine whether chitosan would prevent *C. albicans* biofilm formation in vivo. We used colony-forming unit counts (Figure 1*A*) and scanning electron microscopic examination (Figure 1*B*–1*G*) to assess infection and biofilm formation. *C. albicans*–infected catheters treated with chitosan had statistically significantly lower microbial burden than did control catheters ($P < .001$) (Figure 1*A*). For instance, there was a 2.5-fold difference in fungal burden between catheters treated with chitosan and the untreated control catheters.

Images of the luminal surface of control or chitosan-treated catheters were taken 24 h after inoculation with *C. albicans* (Figure 1*B*–1*G*). *C. albicans* formed extensive biofilms in untreated control catheters, consisting of a network of hyphae and yeast cells embedded in a heterogeneous matrix (Figure 1*B*–1*D*). Conversely, *C. albicans* biofilms did not form on chi-

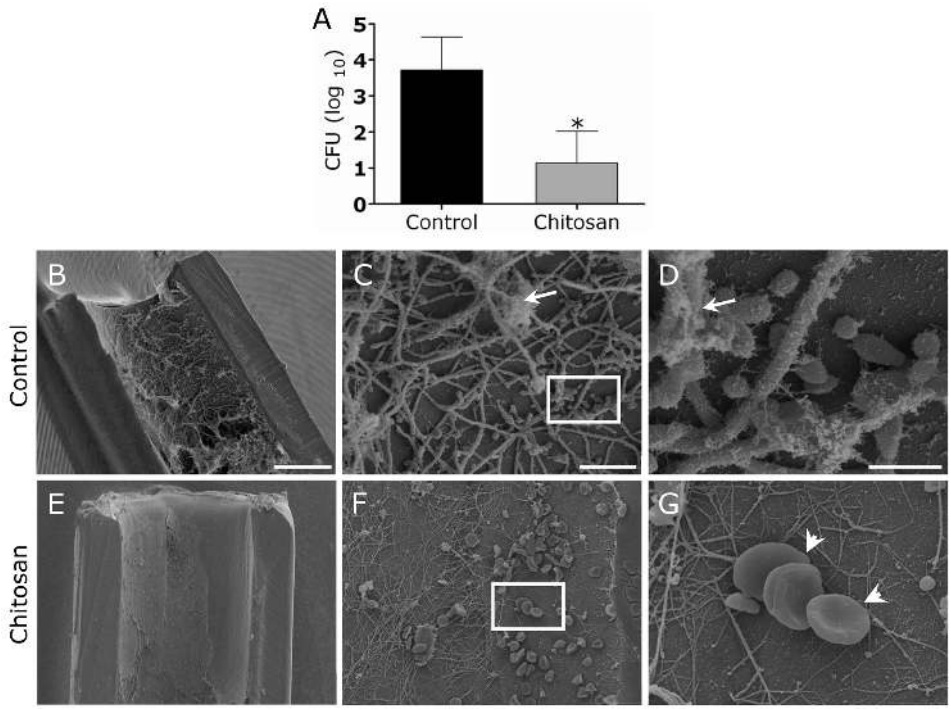

Figure 1. Reduction of *Candida albicans* SC5314 surface-associated growth by chitosan-coated catheters. *A*, Mean fungal burden in catheters infected with 1×10^6 cells/μL *C. albicans*. The fungal burden in chitosan-treated catheters was statistically significantly lower than that in untreated control catheters. Error bars denote the standard deviations. **P* < .001 for comparison between the untreated and chitosan-treated groups. This experiment was performed twice, with similar results each time. *B–D*, Scanning electron microscopic examination of untreated *C. albicans* biofilms; *E–G*, scanning electron microscopic examination of *C. albicans* biofilms treated with 5 mg/mL chitosan. *C. albicans* biofilm formed on the luminal surface of the untreated catheter (*B*). Biofilms grown in the absence of chitosan showed a network comprising yeast cells and hyphae surrounded by moderate amounts of exopolymeric matrix (*C*). *D*, Higher magnification of the boxed region in panel *C*. White arrows indicate exopolymeric matrix. Chitosan-treated catheters did not form *C. albicans* biofilm (*E*). Instead, they showed minimal adhesion of red blood cells and fibrous debris (*F*). *G*, Higher magnification of the boxed region in panel *F*, with white arrowheads indicating red blood cells. Scale bar for panels *B* and *E*, 200 μm; scale bar for panels *C* and *F*, 20 μm; scale bar for panels *D* and *G*, 5 μm. CFU, colony-forming unit.

tosan-treated catheters. Instead, images showed rare yeast-like cells and many adherent red blood cells and fibrous debris (Figure 1*E*–1*G*).

We used ζ potential analysis to explore whether a subinhibitory concentration of chitosan would disrupt fungal cell membranes as yeasts settled on the colonizing surface, resulting in inhibited biofilm formation (data not shown). The negative charge of *Candida* species (charge of *C. albicans*, -25.09 ± 1.27 mV; charge of *C. parapsilosis*, -36.55 ± 1.67 mV) was statistically significantly ($P < .001$) altered to a net positive charge after exposure to 0.16 mg/mL chitosan (charge of *C. albicans*, 68.26 ± 1.24 mV; charge of *C. parapsilosis*, 59.29 ± 0.54 mV).

We investigated the antifungal efficacy of chitosan against *Candida* species biofilms in vitro. Biofilms of *C. albicans* and *C. parapsilosis* treated with chitosan showed a statistically significant reduction in metabolic activity, compared with untreated biofilms (Figure 2*A*). For example, the metabolic activity of *Candida* species biofilms was reduced ~70% after 0.5 h of treatment with 2.5 mg/mL chitosan ($P < .001$). To confirm the results obtained with the XTT assay, the percentage of survival of the cells in the biofilm was determined (Figure 2*B*). *Candida* species biofilms were significantly susceptible (~50% killing) to chitosan after 0.5 h of treatment with 0.625 mg/mL chitosan, and >95% killing was achieved with 2.5 mg/mL chitosan ($P < .001$). The minimum inhibitory concentration at which 50% of planktonic yeasts was inhibited, as determined by method M27-A of the Clinical and Laboratory Standards Institute, was 0.312 mg/mL (data not shown).

Scanning electron microscopic examination was used to determine the architectural differences between untreated and chitosan-treated *C. albicans* biofilms (Figure 2*C* and 2*F*). Untreated *C. albicans* biofilms comprised a dense network of yeast cells and hyphae surrounded by moderate amounts of exopolymeric matrix (Figure 2*C*). In contrast, biofilms coincubated with chitosan displayed enlarged, deformed, and damaged yeast cells that lacked exopolymeric matrix (Figure 2*F*).

Confocal microscopic examination was used to correlate the XTT reduction and colony-forming unit killing assay results with the visual effects on biofilm metabolism and structure (*C.*

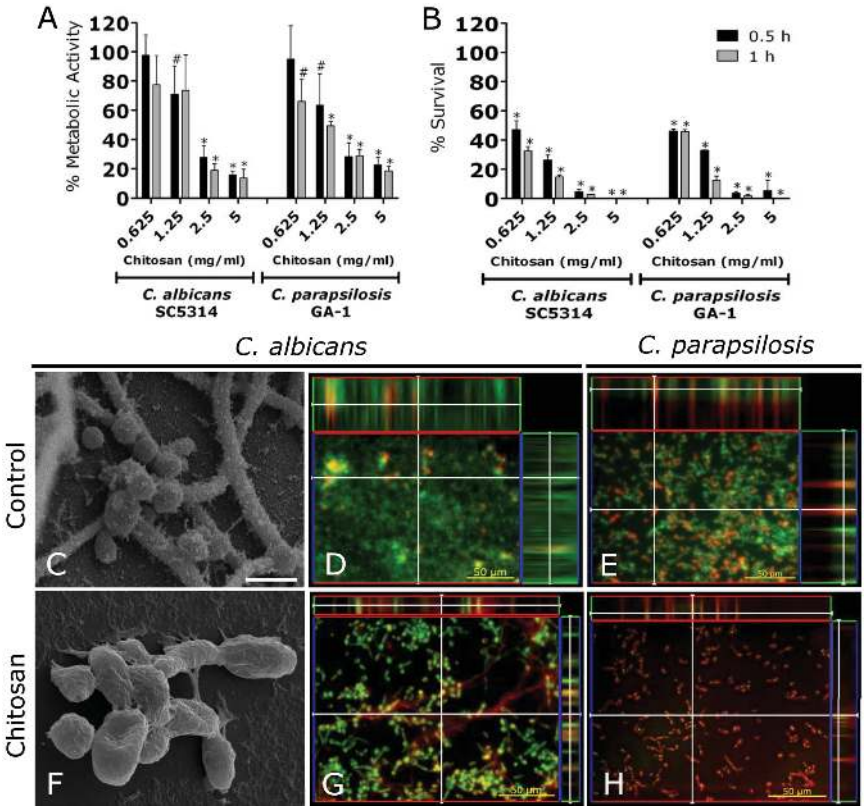

Figure 2. Susceptibility of *Candida* species biofilms to chitosan. The percentage of metabolic activity of untreated and chitosan-treated *Candida albicans* SC5314 and *Candida parapsilosis* GA-1 biofilms was measured by means of the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H–tetrazolium hydroxide reduction assay (*A*). The percentage of survival of untreated and chitosan-treated *C. albicans* and *C. parapsilosis* biofilms was measured by determination of the numbers of colony-forming units (*B*). Biofilms were exposed to chitosan for 0.5 or 1 h, and their metabolic activity levels and survival percentages were compared with those of biofilms incubated in phosphate-buffered saline (control). Importantly, the concentrations of chitosan that were used to evaluate biofilm susceptibility were not toxic to human endothelial cells (data not shown). Data are presented as the means of 4 measurements, and error bars denote standard deviations. $^{\#}P < .05$ and $^{*}P < .001$ for comparison between the untreated and chitosan-treated groups. These experiments were performed twice, with similar results each time. *C*, *F*, Scanning electron microscopic examination of untreated *C. albicans* SC5314 biofilms and *C. albicans* SC5314 biofilms treated with chitosan, respectively. Biofilms grown in the absence of 1.25 mg/mL chitosan demonstrated a dense network of yeast cells and hyphae surrounded by moderate amounts of exopolymeric matrix. In contrast, biofilms coincubated with chitosan displayed enlarged and deformed yeast cells that lacked exopolymeric matrix and were visibly damaged. Scale bar for panels *C* and *E*, 5 μm. *D*, *G*, Confocal microscopic examination of untreated *C. albicans* biofilms and *C. albicans* biofilms treated with chitosan, respectively. *E*, *H*, Confocal microscopic examination of untreated *C. parapsilosis* biofilms and *C. parapsilosis* biofilms treated with chitosan, respectively. Orthogonal images of mature fungal biofilms showed metabolically active cells (FUN-1 staining, *red*) embedded in the polysaccharide extracellular material (concanavalin A-alexa fluor 488 conjugate staining, *green*); the yellow-brownish areas represent metabolically inactive or nonviable cells. Images were obtained after 1 h of exposure of the fungal cells to 1.25 mg/mL chitosan and were compared with those of biofilms incubated in presence of phosphate-buffered saline. The pictures were taken at ×40 power. Scale bars for panels *D*, *E*, *G*, and *H*, 50 μm. The results are representative of 2 separate experiments.

albicans, Figure 2*D* and 2*G*; *C. parapsilosis*, Figure 2*E* and 2*H*). Regions of red fluorescence (FUN-1) represent metabolically active cells, green fluorescence (ConA) indicates glucose and the mannose residues of cell walls, and yellow-brownish areas represent metabolically inactive or nonviable cells. *Candida* species biofilms grown in the presence of PBS alone showed regions of high metabolic activity (Figure 2*D* and 2*E*). Fungal biofilms treated with 1.25 mg/mL chitosan manifested a decrease in the thickness of the exopolymeric matrix and a decreased level of metabolic activity (Figure 2*G* and 2*H*).

Discussion. *Candida* species have emerged as major agents of hospital-acquired infections. They are ranked as the fourth most commonly isolated bloodstream pathogens [3], with *C. albicans* and *C. parapsilosis* being highly associated with catheter infections [5, 10]. We used a previously characterized CVC model to mimic device-associated infection to determine whether chitosan would prevent *C. albicans* biofilm formation in vivo. Our results showed that chitosan-coated catheters inhibit *C. albicans* biofilm formation in vivo. A recent study showed that chitosan-coated surfaces have antibiofilm

properties against bacteria and fungi in vitro, including *C. albicans* [7]. This phenomenon has been attributed to the ability of cationic chitosan to disrupt negatively charged cell membranes as microbes settle on the surface [6]. In this regard, our ζ potential analysis demonstrates that chitosan has a profound effect on the negative charge of the fungal cellular membrane, which may translate into interference with surface colonization or adhesion and cell-cell interactions during biofilm formation [11]. For example, a net positive charge of the fungal surfaces may keep yeast cells in suspension, preventing biofilm formation [12]. Likewise, alterations in cell charge may lead to increased phagocytosis and killing of fungal cells by macrophages [13].

Chitosan decreased the metabolic activity and survival of *Candida* species biofilms. This phenomenon may be caused by physical stress on the biofilm structure due to permeabilization of the cellular membrane, which allows increased penetration of chitosan and effective delivery of its antifungal activity [6, 14]. Binding of chitosan to DNA and inhibition of messenger RNA synthesis occurs through chitosan penetration toward the nuclei of the microorganisms and interference with the synthesis of messenger RNA and proteins [14]. It is most likely that the interaction between positively charged chitosan molecules and negatively charged microbial cell membranes leads to the leakage of proteinaceous and other intracellular constituents, causing cell death [15].

The findings of this study suggest that chitosan might offer a flexible, biocompatible platform for designing coatings to protect surfaces from infection. To our knowledge, this is the first report showing that chitosan inhibits candidal biofilm formation in vivo. Furthermore, we demonstrated that chitosan exhibits strong antifungal activity against *Candida* species biofilms in vitro. Our data suggest that chitosan could potentially be developed as an antimicrobial agent in the treatment of catheter-related fungal biofilm diseases.

References

1. Crnich CJ, Maki DG. The promise of novel technology for the prevention of intravascular device–related bloodstream infection. I. Pathogenesis and short-term devices. Clin Infect Dis **2002**; 34:1232–1242.
2. Rex JH, Bennett JE, Sugar AM, et al. Intravascular catheter exchange and duration of candidemia. Clin Infect Dis **1995**; 21:994–996.
3. Pappas PG, Rex JH, Sobel JD, et al. Guidelines for treatment of candidiasis. Clin Infect Dis **2003**; 38:161–189.
4. Donlan RM. Biofilms and device-associated infections. Emerg Infect Dis **2001**; 7:277–281.
5. Kuhn DM, Ghannoum MA. *Candida* biofilms: antifungal resistance and emerging therapeutic options. Curr Opin Investig Drugs **2004**; 5: 186–197.
6. Rabea EI, Badawy ME, Stevens CV, Smagghe G, Steurbaut W. Chitosan as antimicrobial agent: applications and mode of action. Biomacromolecules **2003**; 4:1457–1465.
7. Carlson RP, Taffs R, Davison WM, Stewart PS. Anti-biofilm properties of chitosan-coated surfaces. J Biomater Sci Polym Ed **2008**; 19:1035–1046.
8. Andes D, Nett J, Oschel P, Albrecht R, Marchillo K, Pitula A. Development and characterization of an in vivo central venous catheter *Candida albicans* biofilm model. Infect Immun **2004**; 72:6023–6031.
9. Meshulam T, Levitz SM, Christin L, Diamond RD. A simplified new assay for assessment of fungal cell damage with the tetrazolium dye, (2,3)-bis-(2-methoxy-4-nitro-5-sulphenyl)-(2H)-tetrazolium-5-carboxanilide (XTT). J Infect Dis **1995**; 172:1153–1156.
10. Trofa D, Gácser A, Nosanchuk JD. *Candida parapsilosis*, an emerging fungal pathogen. Clin Microbiol Rev **2008**; 21:606–625.
11. Miyake Y, Tsunoda T, Minagi S, Akagawa Y, Tsuru H, Suginaka H. Antifungal drugs affect adherence of *Candida albicans* to acrylic surfaces by changing the zeta-potential of fungal cells. FEMS Microbiol Lett **1990**; 57:211–214.
12. Savard T, Beaulieu C, Boucher I, Champagne CP. Antimicrobial action of hydrolyzed chitosan against spoilage yeasts and lactic acid bacteria of fermented vegetables. J Food Prot **2002**; 65:828–833.
13. Nosanchuk JD, Casadevall A. Cellular charge of *Cryptococcus neoformans*: contributions from the capsular polysaccharide, melanin, and monoclonal antibody binding. Infect Immun **1997**; 65:1836–1841.
14. Sudarshan NR, Hoover DG, Knorr D. Antibacterial action of chitosan. Food Biotechnol **1992**; 6:257–272.
15. Jung B, Kim C, Choi K, Lee YM, Kim J. Preparation of amphiphilic chitosan and their antimicrobial activities. J Appl Polym Sci **1999**; 72: 1713–1719.