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Comparative study of preparation, characterization and anticandidal activities of chitosan silver nano composite (CAgNC) compared with low molecular weight chitosan (LMW-chitosan)

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Abstract

Chitosan-silver nanocomposite (CAgNC) was green synthesized using low molecular weight chitosan (LMW-chitosan) and silver nitrate without applying external chemical-reducing agents. The newly synthesized CAgNC was characterized by UV–visible spectroscopy, fourier transform infrared spectroscopy (FT-IR), X-ray diffraction (XRD), field emission electron microscopy (FE-SEM and FE-TEM), inductively coupled plasma–atomic emission spectroscopy (ICP–AES), particle size and zeta potential analysis. The average size of LMW-chitosan and CAgNC were 1776 ± 23 nm and 240.1 ± 23.6 nm, respectively. The zeta potential of CAgNC was observed as + 41.1 mV**.** The AgNPs which are deposited on chitosan matrix had average size ranges between 5-50 nm. The Ag content of the CAgNC was determined as 0.696± 0.054% (w/w). The minimum inhibitory concentration (MIC) values of LMW-chitosan and CAgNC against *Candida albicans* were determined as 100 and 50 µg/mL, whereas the minimum fungicidal concentration (MFC) values were recorded as 400 and 150 µg/mL, respectively. Propidium iodide (PI) uptake results suggested that CAgNC has affected to permeability of cell membrane of *C. albicans.* Moreover, CAgNC induced the level of reactive oxygen species (ROS) at higher level when compared to the LMW-chitosan in concentration dependent manner. This report illustrates the eco-friendly approach for the reduction of silver ions using LMW-chitosan as a reducing agent to make biologically active composite (CAgNC) and as potential antifungal agent against *C. albicans.*

Keywords: Low molecular weight chitosan (LMW-chitosan); chitosan silver nano composite (CAgNC); Silver nano particles (AgNPs); antifungal agent; *C. albicans.*

1. Introduction

Chitosan, a natural cationic polysaccharide which is consisted of co-polymers of glucosamine (β 1–4-linked 2-amino-2-deoxy-d-glucose) and N-acetyl glucosamine (2-acetamido-2-deoxy-d-glucose). Chitosan is derived from partial deacetylation of chitin obtained from crustaceans or the mycelium of fungi.[1] Bacteriostatic [2] and fungistatic effects[3] due to reactive amino groups and metal ion chelating activity associated with linear polyamine (poly- D-gulcosamine) structures are the main functional properties of chitosan. Recently, applications of chitosan have extended to various fields such as medicine, food, chemical engineering, pharmaceuticals, nutrition, environmental protection and agriculture.[4] In particular, the antifungal and antibacterial activities of chitosan have been investigated against wide range of pathogenic strains.[5] The size and zeta potential of chitosan particles are critical properties when considering its bioactivities.[6]

Nanosilver (silver nanoparticles, AgNPs, or Ag^0_{nano}) is considered as zero valent silver (Ag^0) having a less than 100 nm of particle diameter. AgNPs are commonly synthesized through chemical reduction methods in which silver salts, such as $AgNO_3$,[7] or silver perchlorate/$AgClO_4$,[8] can be reduced by reducing agent like glucose,[7] or sodium borohydride/$NaBH_4$.[9] In function, AgNPs displays stronger, longer-term, and broader spectrum of antimicrobial activities when compare with other metallic nano particles.[10] Meanwhile coating agents or stabilizers, such as polysaccharides,[11] poly vinyl alcohol/PVA,[12] poly ethylene glycol/PEG,[13,14] or citrate,[15] are generally used to prevent aggregation of AgNPs. To achieve better biomedical performances AgNPs, many researches have tested polymer based composite materials combined with AgNPs.[16]

Moreover, polymer embedded AgNPs have been shown superior characteristics such as longer stability, better dispersion and low toxicity levels. Chitosan-silver nano composite

(CAgNC) is one of the composite materials which can be synthesized via electrochemical,[17] chemical,[18] green synthesis,[19,20] and biosynthesis methods.[21] It possesses antimicrobial activity, [22,23] bio sensing potential,[22] and dye oxidation properties [24]. In recent years, severe fungal infections have caused increasing morbidity and mortality among immunocompromised patients who need intensive treatments.[25] *C. albicans* is the most widespread species among other Candida species such as *C. tropicalis, C. glabrata, C. kruisei.*[26] Therefore, it is an urgent need for development of new and non-toxic antifungal agents against, *C. albicans.*

Our main objective of the present study was to compare the physio-chemical properties and anticandidal properties of CAgNC with its precursor LMW-chitosan. For that we firstly prepared the CAgNC using LMW-chitosan and determined the physiochemical properties such as particle size, zeta potential, UV-vis absorption, FE-SEM, FE-TEM and XRD. In order to make functional comparison, antifungal activity against *C. albicans* was assessed under various parameters such as MIC, MFC, cell viability, change of cell membrane structure, capacity of ROS production and PI uptake. Based on the results and interpretation of possible mode of action, we conclude that newly synthesized CAgNC has superior antifungal activities than LMW-chitosan.

2. Experimental section

2.1 Synthesis and characterization of CAgNC from LMW-chitosan

CAgNC was green synthesized by reduction method using LMW(50-150 kDa)-chitosan with a deacetylation degre of ~85% (Sigma–Aldrich, USA). In brief, the CAgNC was synthesized by adding 4 mL of freshly prepared 0.01 M $AgNO_3$ solution (Sigma Aldrich, USA) followed by addition of 400 µL of 0.5 M NaOH solution (Biosesang, Korea) to 100 ml of 0.2% (w/v) LMW-chitosan solution with constant stirring at 95 °C. The formation of AgNPs was

indicated by the appearance of a yellow color about 1 min after the addition of the NaOH solution. After 15 min, the resulting suspension was filtered and washed several times using distilled water and then dried at 60 ^{0}C for 6 h. In order to confirm the formation of AgNPs, UV−Vis spectroscopy was carried out using double beam UV−vis spectrophotometer (Mecasys, Korea), over a range of 300- 800 nm. The percentage of Ag in CAgNC was determined using an ICP–AES (Perkin-Elmer Optima, USA). FT-IR spectra was recorded in the wavelength region 4000-600 cm^{-1} using Bio-Rad 175 C FTS spectrophotometer in Attenuated Total Reflectance (ATR) mode. The surface morphology was examined by FE-SEM analysis (Hitachi S-4800, Japan) operating at an accelerating voltage of 3.0 kV. The particle size and shape of the CAgNC was analyzed using FE-TEM, (Model Tecnai G2 F30 S-Twin, FEI, USA) operating at 300 keV. The phase analysis was done by observing the SAED pattern to confirm the crystal structure of CAgNC. X-ray diffraction (XRD) is a versatile, non-destructive analytical method for the identification and quantitative determination of various crystalline phases. Powder XRD analysis was conducted via Philips PW 1710 diffractometer with Cu Kα radiation (λ = 1.5406 Å) and graphite monochromator, operated at 45 kV; 30 mA and 25 °C. Particle size distribution and zeta potential of CAgNC and LMW-chitosan were determined by Zetasizer S-90 Malvern instruments (Malvern, UK) using diluted and dispersed solution of CAgNC in 0.25 % (V/V) acetic acid.

2.2 Analysis of anticandidal activities of CAgNC and LMW-chitosan

MIC and MFC of CAgNC and LMW -chitosan against *C. albicans* were determined via turbid metric assay as described previously.[28] The different concentrations of CAgNC and LMW-chitosan (25, 50, 75, 100, 150 and 200 µg/mL) were added to 4 mL of potato dextrose broth (PDB, Difco-USA) with *C. albicans* at 0.05 OD: 600 nm (10^5 CFU/mL) and incubated at

120 30 ^{0}C while shaking at 150 rpm for 24 h. The corresponding control test was carried out without
121 CAgNC and LMW-chitosan, whereas the positive control was conducted with 10 µg/mL
122 Nystatin. All experiments were carried out in triplicates.
123
124 **2.3 Determination of ROS production and cell viability**
125 To determine the ROS production and the cell viability in *C. albicans* culture (0.05 OD,
126 600nm) with different concentrations of CAgNC and LMW-chitosan (0 to 100 µg/mL) was kept
127 in a shaking incubator for 6 h at 30 ºC. ROS generated cells were stained with 30 µg/mL 5-(and-
128 6)-carboxy-2',7' dichloro dihydro fluorescein diacetate (H_2DCFDA) followed by 30 min
129 incubation and harvesting by centrifugation at 13000 rpm for 2 min. Cells were washed and
130 dissolved using ×1 PBS to quantify ROS generation using the FACScaliber flow cytometer
131 (Becton Dickinson, USA). Cell viability was determined by MTT assay. Briefly, after 24 h
132 incubation period, the samples were treated with 70 µg/µL of MTT solution (3-(4, 5-dimethyl-2-
133 thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide) and incubated for additional 30 min. Harvested
134 cells were re-suspended in DMSO (200 µg/µL well^{-1}) and cell viability was detected at OD 570
135 using a micro plate reader (Thermo, USA) attached to a computer.
136
137 **2.4 Effect on CAgNC and LMW-chitosan on plasma membrane of *C. albicans* by PI assay**
138 Cell membrane integrity of CAgNC and LMW-chitosan treated *C. albicans* was assessed
139 by monitoring the uptake of the fluorescent probe, PI (Sigma Aldrich, USA). For the
140 determination of the PI uptake, cell suspensions of the control, MIC and MFC levels treated
141 samples were centrifuged (3500 rpm, 2 min,) and the pellets were re-suspended in PBS. The
142 treated cells were incubated with PI (5 µg/mL) at 30 ^{0}C for 15 min in dark. Over staining were

washed twice with PBS. Finally, one drop of each suspensions was placed on the cover slip and observed using a Zess LSM 510 meta confocal laser scanning microscope (CLSM) scan head integrated with the Axiovert 200 M inverted microscope (Carl Zeiss, Jena, Germany). *C. albicans* cells were observed through a 40 x 1.3 oil objective and PI was excited with the 543 laser line and the emission was recorded through a 585 long-pass filter.

2.5 Statistical analysis

All the data related to the cell viability was illustrated as means ± SD for triplicate reactions. Statistical analysis was performed using unpaired, two-tailed *t*-test to calculate the *P*-value using GraphPad program (GraphPad Software, Inc.). The significant difference was defined at $P < 0.05$.

3. Result and discussion

3.1 Synthesis and characterization of CAgNC

Present study describes the use of LMW-chitosan with AgNPs to develop biologically active and superior anticandidal agent against *C. albicans.* The first part of this work is synthesis of CAgNC using LMW-chitosan and physiochemical characterization. The progress of the AgNPs synthesis was tracked by using UV−Vis spectroscopy. The UV–visible absorption spectra of LMW-chitosan and CAgNC are shown in Fig. 1. The spectra exhibited an absorption band around 415 nm for CAgNC. However, there is no specific absorption spectrum observed in LMW-chitosan. The surface plasmon resonance (SPR) band of spherical AgNPs was observed around 420 nm and it clearly evidences for the formation of AgNPs as previously reported.[29] To convert the Ag^+ into metallic Ag, an electron supplier or a reducing agent should be added. When NaOH is added

to an $AgNO_3$ aqueous solution, the pH of the solution can be increased and Ag_2O is precipitated as solid mass.[30] However, in this study, a solid gray precipitate of Ag_2O was not formed because Ag+ stabilized by the basic chitosan suspension. Then Ag+/chitosan complex has allowed Ag+ to collect electrons from the basic suspension and to be reduced to an Ag atom. Twu et al[20], has suggested the greater probability of suppling electron by degradation products of low-molecular weight chitosan (e.g. glucosamide) and functioning as a reducing agent.

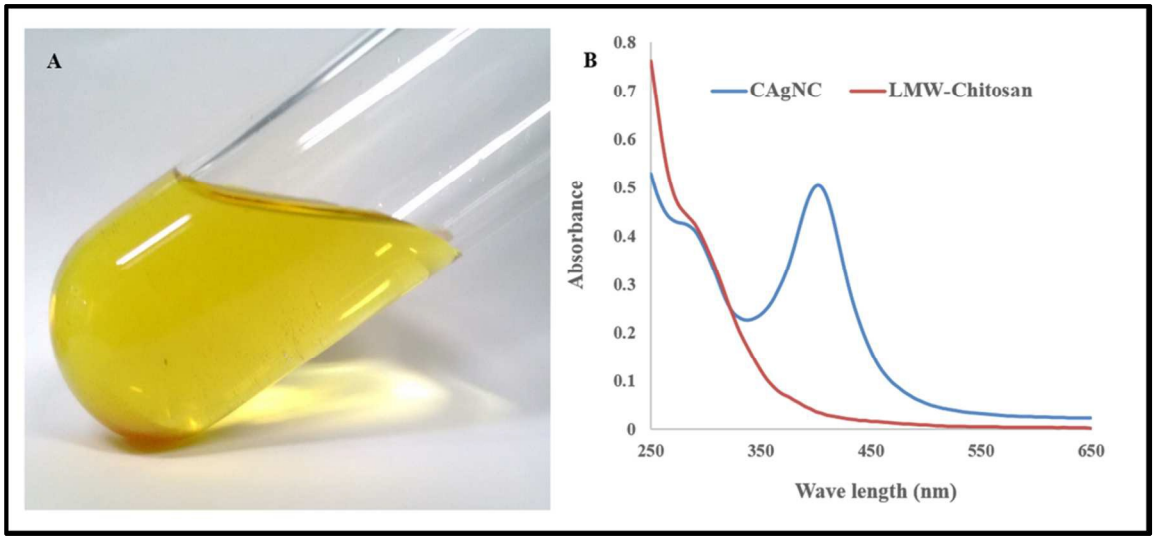


Fig 1. Product of CAgNC and UV–visible spectroscopy analysis. (A) Formation of AgNPs on chitosan matrix which develops yellow color. (B) UV-Vis spectrum of AgNPs presenting an absorption peak at 410 nm due to surface plasma resonance (LMW-Chitosan as precauser).

We studied the chemical interaction between LMW-chitosan and Ag in the CAgNC matrix by FT-IR spectral analysis. Results shows the FT-IR spectrum of CAgNC indicating the band at 3366 cm−1 which confirm the stretching vibrations of -OH and -NH groups (Fig. S1). Moreover, additional bands were displayed at 2871 cm−1, 1645 cm−1, 1375 cm−1, 1060 cm−1 which are ascribed to the asymmetric stretching vibrations of -CH group, amide group (C-O stretching

along-N-H deformation), COO− group carboxylic acid salt, and stretching vibrations of C-O-C in the glucose unit, respectively. The LMW-chitosan shows all the corresponding bands of CAgNC. However, the spectrum of the CAgNC was shifted towards lower wave numbers (amine group was shifted from 1658 cm^{-1} to 1645 cm^{-1}) when compare with the spectrum of LMW-chitosan. This suggests the attachment of Ag into N atoms (amino groups), which reduces the vibration intensity of the N–H bond due to the greater molecular weight of CAgNC due to the incorporation of Ag atoms in to LMW-chitosan as described previously.[31]

The surface morphology of the synthesized CAgNC and LMW-chitosan was analyzed using FE-SEM and images are presented in Fig. 2 A & B. The FE-TEM image (Fig. 2 C) implies the presence of spherical AgNPs in the chitosan suspension. Also, it clearly indicated that AgNPs were deposited on chitosan matrix and the average particle size of AgNPs lies between 5-50 nm. The three *diffraction* patterns observed in the selected area of electron diffraction (SAED) pattern are shown in Fig. 2 D, and it can be indexed to a face centered cubic lattice. The first strongest ring is the combination of both (111) and (200) planes, whereas the second ring corresponds to the crystallographic plane of (220). The third ring represents the (311) plane of Ag. The SAED pattern was completely aligned with the XRD pattern.

XRD is a versatile and non-destructive analytical method for the identification and quantitative determination of various crystalline phases. The structural properties of CAgNC were analyzed using XRD technique. XRD analysis results revealed that pattern of CAgNC was clearly differed from that of LMW-chitosan (Fig. 3). The peak for LMW-chitosan was appeared at 2θ value of the broad peak around 5°–25° (Fig. S2). The XRD pattern of powdered CAgNC showed Bragg reflections with 2θ values of 38.12, 44.22, 64.36 and 77.32 for a set of lattice planes which could be indexed to (1 1 1), (2 0 0), (2 2 0) and (3 1 1) planes of face centeredcubic

geometry of Ag and the existence of broad peak between 5°–25° which can be attributed to the presence of LMW-chitosan in the CAgNC. The results showed that the synthesized CAgNC contains AgNPs in crystalline structure, since the position and the relative intensity of all the diffraction peaks of the samples were consistent with the crystalline pattern of Ag.[32] The lattice parameters were determined to be a = 4.0580 that matches with the Joint Committee on Powder Diffraction Standards (JCPDS) file no. 87– 0720. There were no additional peaks in the spectra, indicating the purity of CAgNC sample and no detectable impurities present.

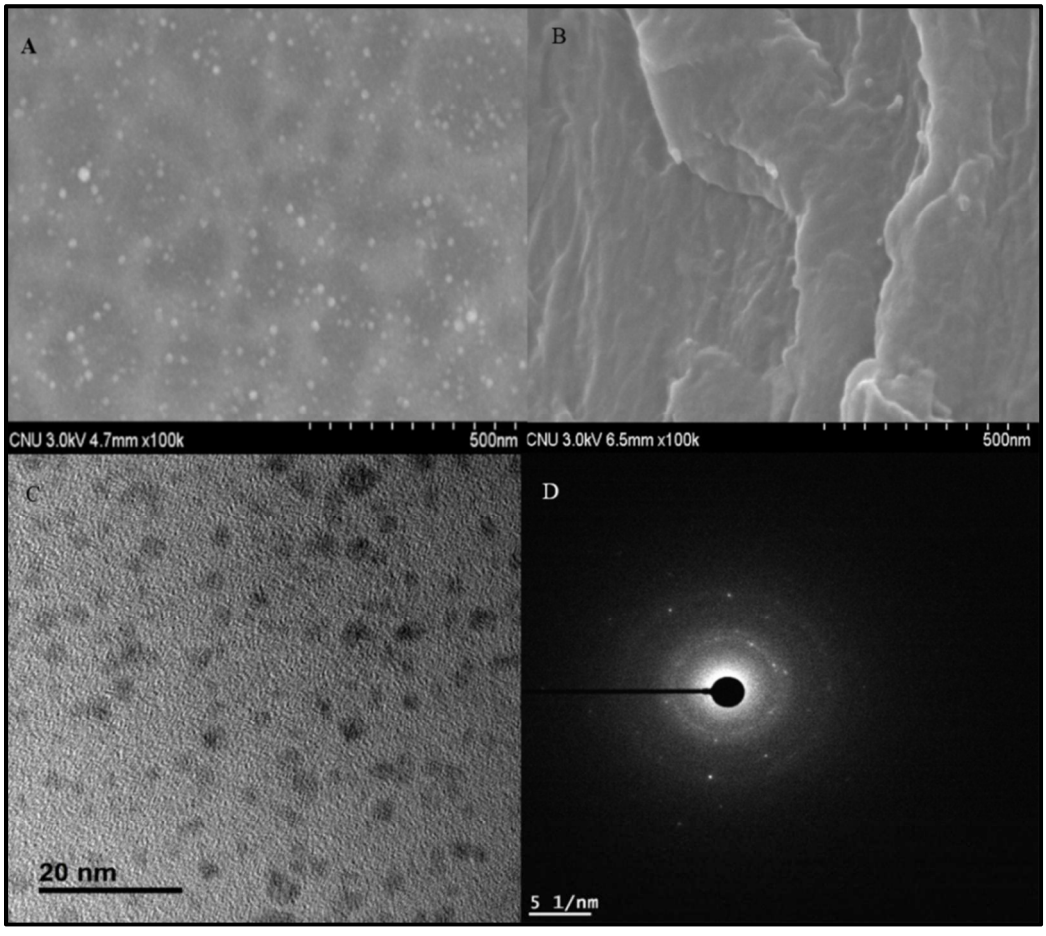


Fig. 2. The FE-SEM and FE-TEM image of CAgNC. (A) FE-SEM image of CAgNC (B) FE-SEM image of LMW-chitosan (C) FE- TEM image of CAgNC (D) SEAD pattern of CAgNC.

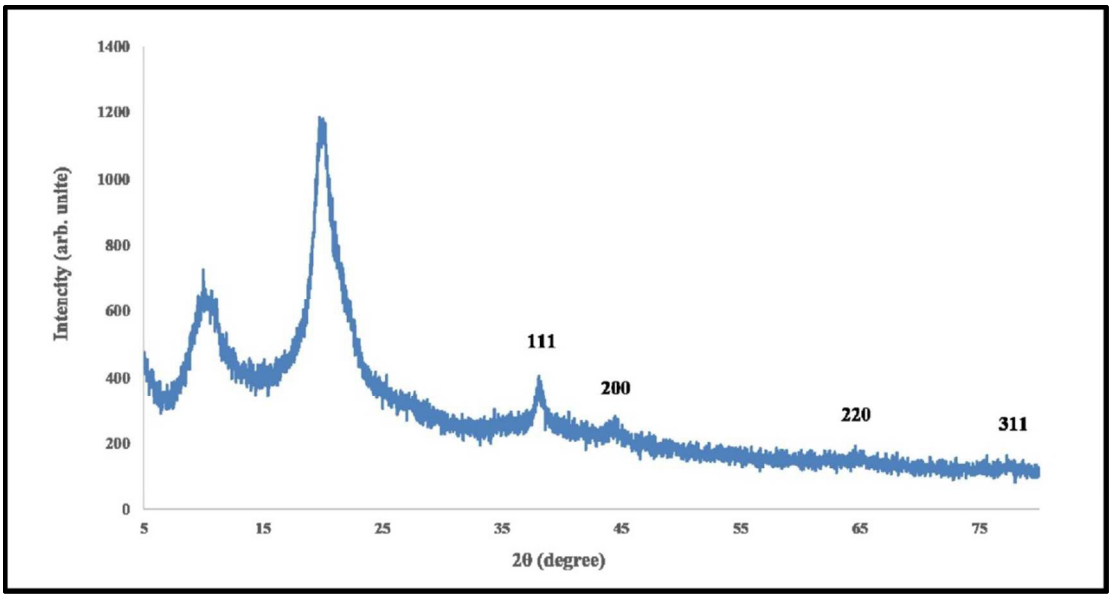


Fig. 3. The XRD graph of the CAgNC.

The particle size distribution of LMW-chitosan and CAgNC was determined using Zetasizer Nano-ZS90. The analysis was performed in triplicates for each sample and presented as mean ± standard deviation (SD) in table 1. Agreeing to the result of this analysis, the average size of LMW-chitosan and CAgNC were 1776 ± 23 nm and 240.1 ± 23.6 nm, respectively (Fig. S3A & S3B). Zeta potential of synthesized CAgNC measured at pH = 4.6 was found as + 41.1 mV (Fig. S3C). The value of zeta potential enables determination of colloid stability and particle aggregation.[33] Therefore, the positive value of the zeta potential of CAgNC could have evidenced the presence of positively charged polymeric layer on AgNPs surface.

3.2 Anticanidal of LMW-chitosan and CAgNC

After characterization of CAgNC, we investigated the antifungal activity against *C. albicans*. The synthesized CAgNC showed superior antifungal activity against *C. albicans* compared to LMW-chitosan. It was found that MIC and MFC of LMW-chitosan as 100 400 µg/mL, respectively (Table 1).

Table 1. Comparison of particle size, zeta potential, MIC and MFC of LMW-chitosan and CAgNC.

Compound name	Particle size (nm) (Mean ± SD)	Zeta potential (mV) (Mean ± SD)	MIC (µg/mL)	MFC (µg/mL)
LMW-chitosan	1776 ± 23.00	-------	100	400
CAgNC	240.1 ± 23.26	+ 41.6 ± 4.64	50	150

Interestingly CAgNC showed significantly lower MIC of 50 µg/mL (2 times lower than LMW-chitosan) and MFC of 150 µg/mL (2.7 times lower than LMW-chitosan), respectively. Ing et al.,[34] showed that LMW-chitosan solution has higher MIC_{90} value (3 mg/ml) compare with chitosan nano particle (0.25 mg/ml) against *C. albicans*. Panacek et al., [25] showed that MIC of stabilized AgNPs varied from 0.052 to 0.84 mg/L with Candida *sp.* The size of particles plays an important role in determination of antimicrobial activity of nanoparticles as they enter the cell walls of microbes through carrier proteins or ion channel and smaller nanoparticles result in a better uptake into microbial cells[6]. Zeta potential has been suggested as a key factor that is contributing to antifungal effect of chitosan through the interaction with negatively charged microbial surface.[35] The synthesized CAgNC has shown higher anticandidal activity because of its low particle size and higher zeta potential value when compare with LMW-chitosan. The mode of action of CAgNC against *C. albicans* is not fully understood and therefor further investigations are required to establish in future.

Recent study[36] suggested that the accumulation of ROS induces and regulates the apoptotic pathway in yeast. Thus, to examine the relationship between the accumulation of ROS and the induction of apoptosis, an experiment was conducted to find out the effect of different concentration of LMW-chitosan and CAgNC on the ROS production and cell viability in *C.*

albicans. ROS level was slightly increased until 75 µg/mL and beyond 100 µg/mL (for LMW, MIC is 100 µg/mL) in LMW-chitosan treated (12.5, 25, 50 and 75 µg/mL) *C. albians.* (Fig. 4). Furthermore, *C. albians* samples which were treated with 12.5 µg/mL and 25 µg/mL concentrations of CAgNC have demonstrated sequentially increased ROS levels. Also, at 50 µg/mL (MIC of 50 µg/mL for CAgNC) a steep increase was observed while it slightly increased again at 100 µg/mL. Moreover similar ROS values were obtained both control and acetic acid treated samples as well as for positive control (10 mM H_2O_2) treated samples. Further, ROS result showed slight increased value for CAgNC treated sample comparing to the LMW-chitosan. The reason for such observation could be that AgNPs have the capacity to inhibit the *C. albians* by increasing the oxidative stress.

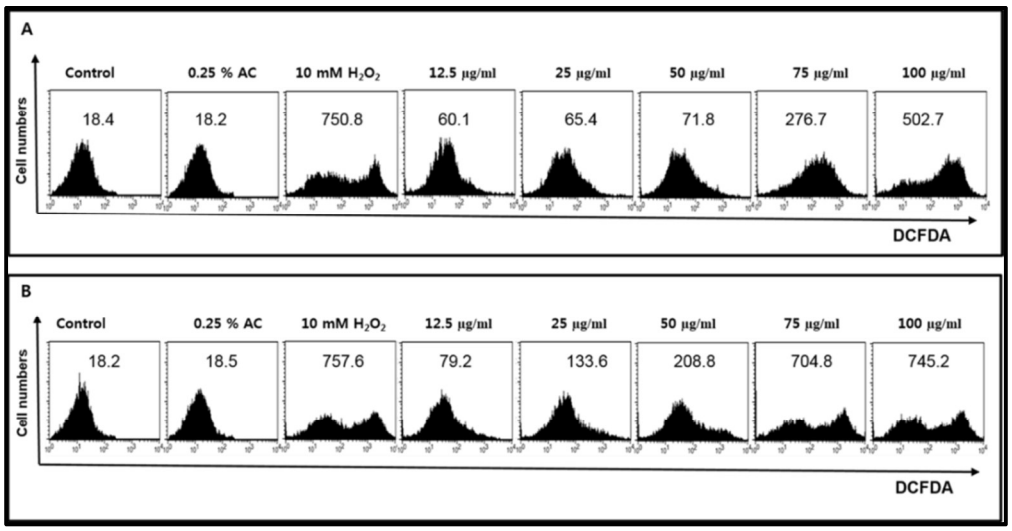


Fig. 4. Effect of LMW-chitosan and CAgNC on ROS production in *C. albicans* cells. A) LMW-chitosan B) CAgNC. 0.25 % (V/V). AC: acetic acid (Negative control), 10 mM H_2O_2 (Positive control).

The overall results suggest that LMW-chitosan and CAgNC trigger the oxidative stress by generating ROS which causes various damages to macromolecules such as DNA, RNA, and proteins as well as other cellular components.[36,37] The production of ROS may be interfered with

the essentials of electronic transport chain which may cause the reduction of cellular energy production.[38] Additionally, the excessive production of ROS may damage plasma membrane and intra cellular organelles which may leads to cell death.[39] The cell viability of *C. albicans* was decreased significantly ($P<0.001$) with increasing the concentration of LMW-chitosan and CAgNC (Fig. 5). Furthermore, highest and lowest cell viability in LMW-chitosan treatment was observed in control and H_2O_2 treated groups, respectively. Whereas highest and lowest cell viability was observed in control and 100 µg/mL of CAgNC treated group, respectively. All the CAgNC treated groups were shown lower cell viability than LMW-chitosan. However, significant difference ($P<0.05$) in cell viability was observed in CAgNC and LMW-chitosan treatments from 12.5 ~100 µg/mL concentration. Thereby, lowest cell viability (28%) was observed in 100 µg/mL CAgNC treatment. Whereas, cell viability for positive control was 34 % at the 10 mM H_2O_2 and negative control 100 % at 0.25 % AC.

The PI uptake result is associated with the occurrence of substantial damage to the membrane, indicating alteration of cell membrane potential, which finally causes cell death. PI could enter the cell and bind to DNA, showing red fluorescence.[40] PI uptake by *C. albicans* cells show concentration dependent mortality in both treated groups where control and MIC treatment have the least number of PI stained *C. albicans* cells which indicates the least number of cell death (Fig. 4 S). However, almost all *C. albicans* cells in both treatments at the MFC level have shown higher red florescence (Fig. 6).

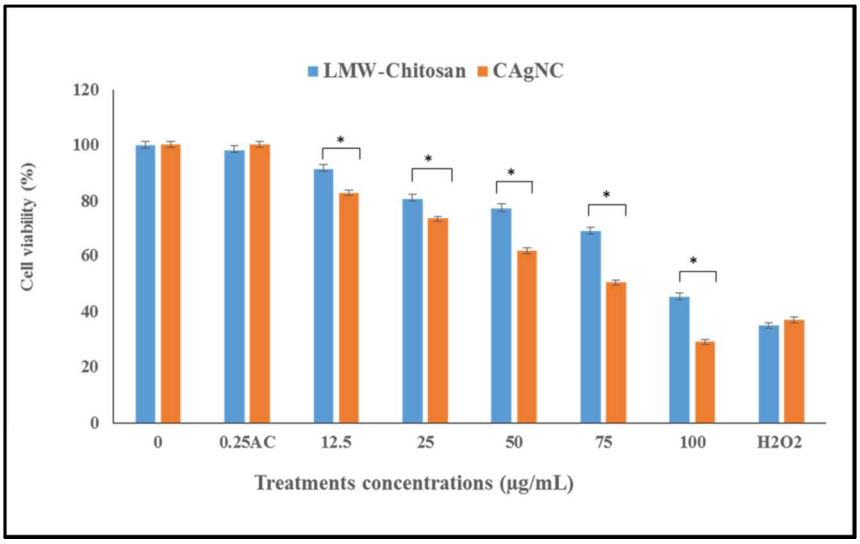


Fig. 5. Comparison of the effect of LMW-chitosan and CAgNC on cell viability of *C. albicans*. Cell viability was assessed by MTT assay (n=3) after treatment with different concentration of LMW-chitosan and CAgNC (12.5 –100 µg/mL). Significant differences in *C. albicans* cell viability were obtained with respect to untreated control ($P \leq 0.05$). The treatments with * mark represent the significant cell viability (%) between LMW-chitosan and CAgNC. Bars with no asterisk were not significantly difference in cell viability. Acetic acid 0.25 % (V/V) as negative control and, 10 mM H_2O_2 as positive control.

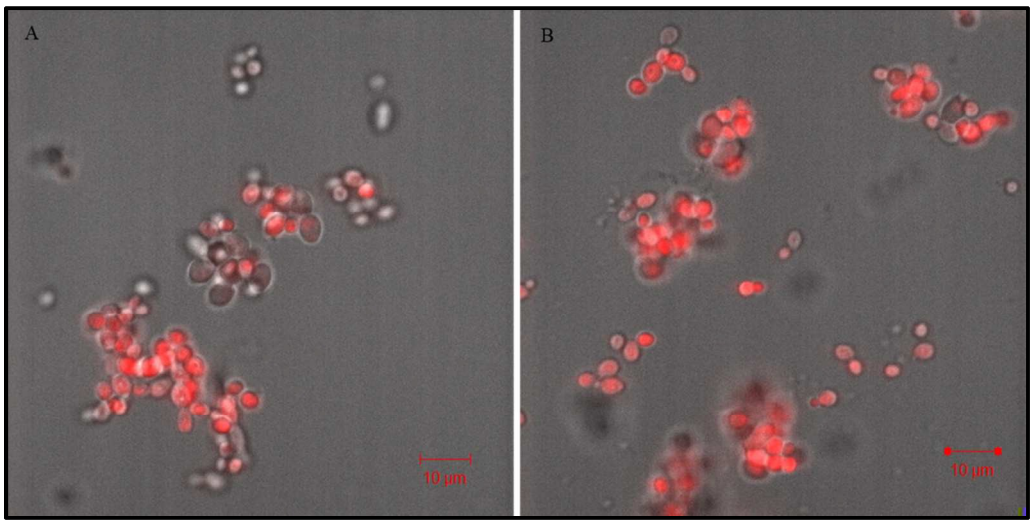


Fig. 6. Effect of LMW-chitosan and CAgNCon cell membrane permeability by PI staning. Merged image of *C. albicans* (by confocal laser scanning microscopy) showing the dead *C. albicans* cells at MFC treatment level. (A) LMW-chitosan (400 µg/mL) (B) CAgNC (150 µg/mL). When cationic chitosan bind to the negatively charge cell surface it may cause to increase hyperpolarization of the plasma membrane.[41] Also, some amount of AgNPs on the surfaces could be ionized and produce cationic silver (Ag^+) traces. This Ag^+ traces flowing in to the cell with the cationic influx generated due to hyperpolarized cell membrane.[42]

4. Conclusions

In summary, we synthesized the CAgNC using LMW- chitosan without external chemical reducing agent and compared their physio-chemical properties and anticandidal action. First we prepared the improved version of chitosan nano composite format with unique characteristics such as smaller partical size (240.1 nm) higher zeta potential (+41.1 mV) and lower amount of AgNPs (0.69%). Moreover, CAgNC had superior anticandidal activities (MIC 50 µg/mL, MFC 100 µg/mL), than the precause LMW-chitosan suggesting that it has great potential to be developed as antifungal agent against wide array of Candida species.

Acknowledgements

This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP) (2014R1A2A1A11054585), research fund of Chungnam National University and part of the project titled 'Development of Fish Vaccines and Human Resource Training', funded by the Ministry of Oceans and Fisheries, Republic of Korea.

References

1. Y. Xia, Q.Fan, D. Hao, J.Wu, G. Ma, and Z. Su, *Vaccine.,* 2015. **33**, 5997–6010.
2. A. Nithya and K. Jothivenkatachalam, *J. Mater. Sci. Mater. Electron.,* 2015, **26**, 10207-10216.
3. A.M.Soliman, S.R. Fahmy and W Mohamed, *J. Basic Appl. Zool.* 2015, in press.
4. S.C. Park, J.P. Nam, J.H. Kim, Y.M.Kim, J.W Nah and M.K.Jang, *Int. J. Mol. Sci*., 2015, **16**, 7995–8007.
5. R. C. Goy, S.T.B. Morais and B.G.A. Odilio, *Rev. Bras. Farmacogn.* 2015, in press.
6. D. Sharma, J. Rajput, B.S. Kaith, M. Kaur and S. Sharma, *Thin Solid Films*, 2010, **519**, 1224–1229.
7. X. Wang, Z.Ji, C.H.Chang, H. Zhang, M. Wang, Y.P. Liao, S. Lin, H. Meng, R. Li and B. Sun, *Small.,* 2014,**10 (2)**, 385-398.
8. Y.Lee and S.G. Oh, *Colloids Surf. A Physicochem. Eng. Aspects.,* 2014, **459**, 172-176.
9. P.L. Freire, T.C. Stamford, A.J. Albuquerque, F.C. Sampaio, H.M. Cavalcante, R.O. Macedo, A. Galembeck, M.A. Flores and A. Rosenblatt, *Int. J. Antimicrob. Agents.,* 2015, **45 (2)**, 183-187.
10. M. Kooti, S. Gharineh, M. Mehrkhah, A.Shaker and H.Motamedi, Chem. *Eng. J.*, 2015. **259**, 34-42.
11. J.E. Skebo, C.M, Grabinski, A.M. Schrand, J.J. Schlager and S.M. Hussain, *Int. J. Toxicol.,* 2007, **26 (2)**, 135-141.
12. P.Khanna, N. Singh, D. Kulkarni, S.Deshmukh, S. Charan and P.Adhyapak, *Mater.Lett.,* 2007., **61 (16)**, 3366-3370.
13. L. Rizzello and P.P. Pompa, *Chem. Soc. Rev.,* 2014, **43(5)**, 1501-1518.

14. P. Simakova , J. Gautier, M. Proch-azka, K. Herve-Aubert and I. Chourpa, *J. Phys. Chem. C.,* 2014, **118 (14)**, 7690-7697.

15. M.N.Croteau, A.D. Dybowska, S.N.Luoma, S.K.Misra and E.Valsami-Jones, *Environ. Chem.,* 2014, 11 (3), 247-256.

16. P. Dubey, B. Bhushan, A. Sachdev,I. Matai,S.U. Kumar and P.Gopinath, *J.Appl.polym.Sci.,* 2015.**132**,42473.

17. F.M.Reicha, A. Sarhan, M.I.Abdel-Hamid, I.M. El-Sherbiny, *Carbohydr. Polym.,* 2012. **89**, 236–244.

18. H. Huang, Q. Yuan, X.Yang, *Colloids Surfaces B Biointerfaces.*, 2004, **39**, 31–37.

19. D.K.Boanic, L. V. Trandafilovic, A.S. Luyt, V.Djokovic, 2010. *React. Funct. Polym.,* 2010,**70**, 869–873.

20. Y.K.Twu, Y.W. Chen and C.M. Shih, *Powder Technol*., 2008, **185**, 251–257.

21. A.M.Youssef, M.S. Abdel-Aziz and S.M.El-Sayed, *Int. J. Biol. Macromol.,* 2014, **69,** 185–91.

22. S. Govindan, E. A. K. Nivethaa, R. Saravanan, V. Narayanan and A. Stephen, *Appl. Nanosci*., 2012, 2, 299–303.

23. H.V.Tran, L.D.Tran, C.T. Ba, H.D.Vu, T.N. Nguyen, D.G. Pham and P.X. Nguyen, *Colloids Surfaces A Physicochem. Eng.* Asp., 2010,**360**, 32–40.

24. J.Santhanalakshmi and V.Dhanalakshmi, *Indian. J. Sci.Tec.*, 2012,**15,** 3834–3838.

25. A.Panacek, M. Kolar, R. Vecerova, R. Prucek, J. Soukupova, V.Krystof, P. Hamal, R. Zboril and L. Kvitek, *Biomaterials.,* 2009, **30**, 6333–6340.

26. S.N.Kulikov, S.A. Lisovskaya, P. V. Zelenikhin, E. A.Bezrodnykh, D.R Shakirova, I. V.Blagodatskikh and V.E.Tikhonov, *Eur. J. Med. Chem*., 2014,**74**, 169–178.

27. S.H.S. Dananjaya , G.I. Godahewa , R.G.P.T. Jayasooriya, J.Lee and M. De Zoysa, Aquaculture., 2016, **450**, 422–430.

28. E. G. Totoli and H. R. N. Salgado, *Pharmaceutics.,* 2015, 7, 106-121.

29. L. S.Wang, C.Y.Wang, C.H.Yang, C.L. Hsieh, S.Y.Chen, C.Y.Shen, J.J. Wang and K.S. Huang. *Int. J. Nanomedicine*., 2015, **10,** 2685–2696.

30. D.K. Bonzanic, L.V. Trandfilovic , A.S Luyt and V. Djokovic, *Reac. Funct. Polymer.,* 2010,**70**,869-873.

31. D.Wei, W.Sun, W.Qian, Y. Ye and X. Ma, *Carbohydr. Res.,* 2009, **344 (17)**, 2375–2382.

32. S.S. Sana, V.R. Badinni ,S.K Arala and V.K.N.Boya, Materials Letters., 2015,**145**,347–350.

33. I. Ostolska and M. Wiśniewska, *Colloid Polym. Sci*., 2014,**292**, 2453–2464.

34. L.Y, Ing, N.M. Zin, A. Sarwar. and H. Katas. *Int. J. Biomater*., 2012. ID/632698.

35. L. C. Chen, S. K. Kung, H.H. Chen and S. B. Lin, *Carbohydr. Polym*., 2010, **82**,913–919.

36. B. Hwang, J.S. Hwang, J. Lee, J.K. Kim, S.R., Kim, Y., Kim and D.G, Lee. *Biochem. Biophys. Res. Commun.,* 2011, **408**, 89–93.

37. C. Zhang, Z. Hu and B.Deng, *Water Res.*, 2016, **88**, 403–427.

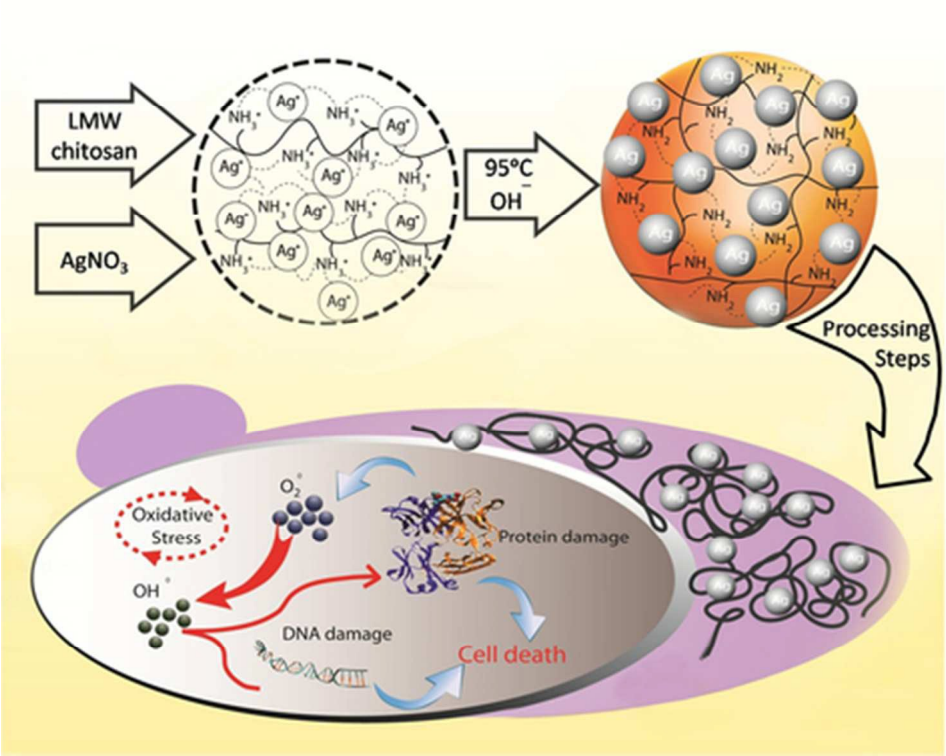
38. E.D.Cavassin, L.F.P. de Figueiredo, J.P. Otoch, M.M.Seckler, R.A. de Oliveira, , F.F.Franco, V.S. Marangoni, , V.Zucolotto, A.S.S. Levin and S.F.Costa, *J. Nanobiotechnology.,* 2015, **13**, 64.

39. G.G.Perrone, S.X.Tan and I.W. Dawes, *Biochim. Biophys. Acta - Mol. Cell Res.,* 2008, **1783**, 1354–1368.

40. A. Banerjee, P.Majumder, S.Sanyal, J.Singh, K.Jana, C.Das and D.Dasgupta, *FEBS Open Bio*., 2014, **4**, 251–9.

41. A. Pena, N.S. Sanchez, M.Calahorra, *Biomed Res. Int.,* 2013, 527-549.

42. A. Ivask, A.Elbadawy,C.Kaweeteerawat,D.Boren, H. Fischer, Z.Ji,C.H.Chang, R.Liu, T.Tolaymat, D.Telesca, J. I.Zink,Y.Cohen, P.A.Holden, and H. A. Godwin, *ACS Nano*., 2014, **8**, 374–386.



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