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The antibacterial properties of a novel chitosan–Ag-nanoparticle composite

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ABSTRACT

Escherichia coli expressing recombinant green fluorescent protein was used to test the bactericidal efficacy of a newly synthesized chitosan–Ag-nanoparticle composite. The composite was found to have significantly higher antimicrobial activity than its components at their respective concentrations. The one-pot synthesis method led to the formation of small Ag nanoparticles attached to the polymer which can be dispersed in media of pH ≤ 6.3. The presence of a small percentage (2.15%, w/w) of metal nanoparticles in the composite was enough to significantly enhance inactivation of *E. coli* as compared with unaltered chitosan. Fluorescence spectroscopy indicated that bacterial growth stopped immediately after exposure of *E. coli* to the composite, with release of cellular green fluorescent protein into the medium at a faster rate than with chitosan. Fluorescence confocal laser scanning and scanning electron microscopy showed attachment of the bacteria to the composite and their subsequent fragmentation. Native protein gel electrophoresis experiments indicated no effect of the composite on bacterial proteins.

1. Introduction

The continuous search for potential antimicrobial agents has lead to identification of antimicrobial biomaterials that are based on polymers or their composites. One such poly-cationic biopolymer with high antimicrobial activity is chitosan, which is composed of polymeric 1 → 4-linked 2-amino-2-deoxy-β-D-glucose. It is prepared by alkaline deacetylation of chitin, which is commonly found in shells of marine crustaceans and cell wall of fungi (Wu et al., 2002; Rabea et al., 2003). Because of its broad spectrum of antimicrobial activity, inherent biodegradability and biocompatibility, and because it is easy to process, chitosan has been used in many applications. The proposed mechanism for its antimicrobial action is binding to the negatively charged bacterial cell wall, with consequent destabilization of the cell envelope and altered permeability, followed by attachment to DNA with inhibition of its replication (Rabea et al., 2003; Helander et al., 2001; Yi et al., 2003; Wang et al., 2004).

It has long been known that silver ions can be bacteriostatic as well as bactericidal (Feng et al., 2000; Mcdonnell and Russell, 1999). The possible use of silver nanoparticles (AgNPs) as antibacterial agent has therefore been investigated as a means of arresting increasing bacterial resistance to conventional bactericides and antibiotics (Gogoi et al., 2006). The proposed mode of action of AgNPs is that they attach to the bacterial cell wall via thiol-containing proteins. They may bind to DNA after penetrating cell membranes with compromised permeability, but recent results suggest that AgNPs do not have any direct effect on either DNA or intracellular protein structures of bacteria.

There is, to the best of our knowledge, only one report (Rhim et al., 2006) on the antimicrobial activity of an AgNP–chitosan nanocomposite. Further investigations of the interactions between AgNP–chitosan composite material and both prokaryotic and eukaryotic cells are needed if useful biocompatible nanocomposite materials with enhanced antimicrobial activity are to be developed.

We have recently developed a novel and 'green' method of synthesis a chitosan–AgNP composite, using chitosan as both the reducing and stabilizing agent (Murugadoss and Chattopadhyay, 2008). The bactericidal effect of the composite was studied using recombinant *Escherichia coli* that expressed green fluorescent protein (GFP). With this model system it was possible to use spectroscopic and microscopic tools to investigate the mechanism of action of the composite.

2. Materials and methods

2.1. Growth media, chemicals and GFP-expressing E. coli

Luria–Bertani broth (LB) and agar powder (bacteriological grade) were purchased from HiMedia, Mumbai, India. Silver nitrate and acetic acid (glacial, 99–100%) were purchased from Merck India Ltd,

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Mumbai, India. Chitosan (high MW, >75% deacetylated) and other high purity molecular biology grade chemicals and reagents for native polyacrylamide gel electrophoresis (PAGE) were obtained from Sigma-Aldrich Chemical Pvt. Ltd., Kolkata, India. The procedure for preparation of the recombinant GFP-expressing *E. coli* cells has been described previously (Gogoi et al., 2006).

2.2. Preparation of the chitosan–AgNP composite

Chitosan–AgNP composite was synthesized by adding 2 ml of freshly prepared 1.0×10^{-2} M $AgNO_3$ solution and then100 μl of 0.3 M NaOH solution to 50 ml of 0.2% (w/v) chitosan solution, with constant stirring, at 95 °C. The appearance of a yellow color about 1 min after addition of the NaOH solution indicated formation of AgNPs. The reaction was stopped after 10 min and the precipitate was filtered and washed with water. The filtrate was dried, and then a 0.2% (w/v) solution of composite in 0.1% (v/v) acetic acid solution was prepared. Various amounts of this composite were used to treat bacteria in the present study, keeping the ratio of the Ag to chitosan concentrations constant at 1:46.

2.3. Characterization of the chitosan–AgNP composite

UV–visible spectra of the composite in 0.1% (v/v) acetic acid were obtained using a spectrophotometer (Lambda-25; Perkin-Elmer, Fremont, CA, USA). The dynamic light scattering (DLS) measurements were performed with a particle size analyzer (LB-550; Horiba, Kyoto, Japan). For transmission electron microscopy (TEM) 5 μl of the composite in LB was drop coated onto a carbon coated copper TEM grid, which was then air-dried. The drop coated grid was analyzed by a high resolution transmission electron microscope (JEM 2100; Jeol, Peabody, MA, USA) operating at an accelerating voltage of 200 KeV.

2.4. Bactericidal activity of the chitosan–AgNP composite

To determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the composite, the GFP-expressing *E. coli* at numbers of 10^6 cfu/ml was inoculated into LB medium supplemented with various concentrations of composite and grown overnight at 37 °C. The minimum concentration of the composite which gave cultures that did not become turbid was taken to be the MIC. The cultures that were not turbid were re-inoculated into fresh LB containing ampicillin at 100 μg ml^{-1}. The MBC of the composite was taken to be the minimum concentration of composite in the initial cultures that gave cultures that did not grow when re-inoculated into LB. Control experiments were performed with acetic acid only. To study the bactericidal activity of the composite, GFP-expressing *E. coli* was grown overnight in 150 ml LB ampicillin medium at pH 6.3. The cells were harvested by centrifugation and resuspended in 300 μl LB. Three 100 μl portions of the cell suspension were inoculated into 50 ml volumes of fresh LB ampicillin media, without the composite or with composite at concentrations of 240 or 360 μg ml^{-1}. During aerobic incubation at 37 °C, the optical densities at 595 nm (OD_{595}) of the cultures were determined using a UV–visible spectrophotometer (SPEKOL 1200, Analytikjena, Jena, Germany); and GFP-expressed fluorescence was determined using a fluorescence spectrophotometer (Varian Cary Eclipse, Palo Alto, CA, USA) with the excitation wavelength set at 400 nm. Numbers of viable *E. coli* were determined by plating serially ten-fold dilutions of bacterial culture on ampicillin supplemented LB-agar plates which were incubated at 37 °C for 24 h.

2.5. Microscopy

The effects of chitosan–AgNP composite on GFP-expressing *E. coli* were monitored using a confocal laser scanning microscope (CLSM; Axiovert 200 M, LSM 510 META, Carl Zeiss, Jena, Germany). Drops (5 μl) of bacterial cultures were placed on microscope slides, air-dried and observed under the microscope. The excitation wavelength was 488 nm while the observation filter had a long-pass filter wavelength above 515 nm. The interactions of the composite with bacterial cells at different times were examined using a scanning electron microscope (SEM; LEO 1430VP, Carl Zeiss). For that a 10 μl drop of each sample was deposited on a glass slide, dried and sputter-coated with gold film in a sputter coater (SC7620 "Mini", Polaron Sputter Coater, Quorum Technologies, Newhaven, England).

2.6. PAGE

For protein analysis by native PAGE, cells from 2 ml portions of control and treated samples were harvested by centrifugation, washed and resuspended in 500 μl of phosphate buffer saline (PBS). The samples were sonicated using a ultra-sonicator (VibraCell, Sonics, Newtown, CT, USA) to disrupt the cells, then centrifuged to remove the cell debris. An 8 μl portion of supernatant from each sample was mixed with 2 μl of 5× sample loading buffer (310 mM Tris–HCl, pH 6.8, 50% (v/v) glycerol, and 0.05% (w/v) bromophenol blue) and loaded onto a 12% (w/v) polyacrylamide gel prepared in 1.5 M Tris–HCl buffer, pH 8.8. Electrophoresis was performed at 20 mA for 3 h using Tris/glycine electrophoresis buffer, pH 8.3 (Tris base, 3 g/l and glycine, 14.4 g/l). Proteins were visualized by staining with Coomasie Brilliant Blue R-250 solution. GFP bands were visualized by UV trans-illumination of the gel.

3. Results

3.1. Preparation and characterization of chitosan–AgNP composite

The UV–visible absorption spectrum of the composite showed a single, narrow and strong peak at around 410 nm, which is characteristic of surface plasmon resonance (SPR) of AgNPs. The selected area electron diffraction pattern (SAED) of small AgNPs confirmed the presence of single crystalline AgNPs. TEM and DLS measurements showed that more than 90% of the particles had diameters between 2 and 4 nm. The composite could be dispersed in 0.1% (v/v) acetic acid and dispersions were stable for at least two months.

3.2. Determination of the antibacterial activity of chitosan–AgNP composite

The effect of the composite on the growth of GFP-expressing *E. coli* was investigated by monitoring culture turbidity (Fig. 1). Growth was completely inhibited at composite concentrations ≥ 100 μg ml^{-1}. This concentration of the composite was considered to be the MIC, while a concentration of 120 μg ml^{-1} was found to be the MBC. Inhibition with

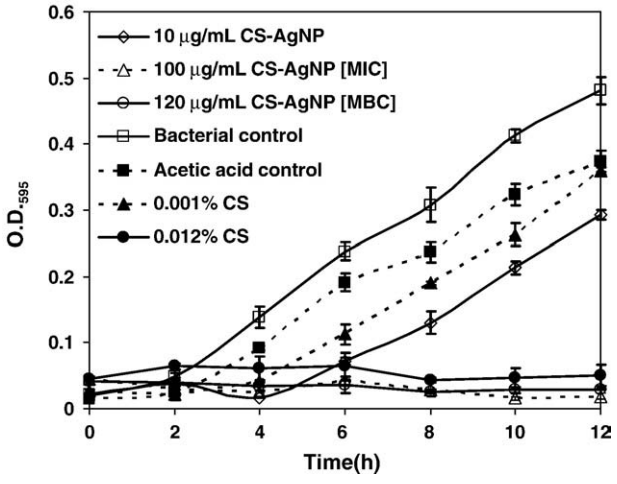

Fig. 1. Effect of different concentrations of chitosan–AgNP composite on the growth of recombinant *E. coli*. CS represents chitosan.

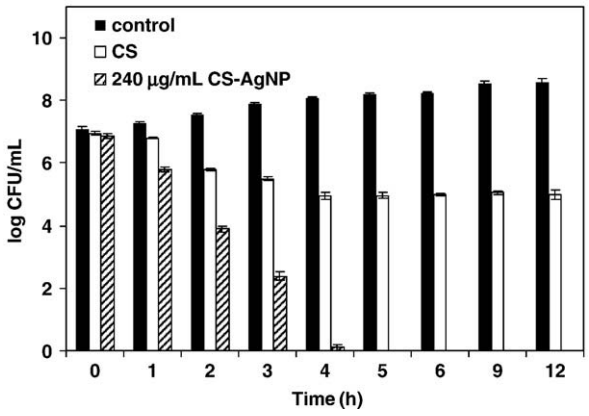

Fig. 2. Comparative effect of CS–AgNPs composite and chitosan alone (with same 0.024% conc. of CS in both the samples) on recombinant *E. coli* viability. CS represents chitosan.

300 ppm acetic acid was negligible. Thus using acetic acid at low concentration for the dispersion of the composite did not have any additional adverse effect on bacterial growth.

Fig. 2 shows the effects of the composite at a concentration of twice the MBC and chitosan alone on the growth of the recombinant bacteria. The composite inactivated all the bacteria within 4 h, whereas chitosan was found to be only bacteriostatic at the test concentration. Moreover, the rate of inactivation by the composite varied proportionally with the composite concentration.

3.3. Mechanism of antibacterial activity of the nanocomposite

Under SEM the composite treated bacteria were seen to be attached to the composite immediately after the treatment (Fig. 3A). By 60 min, fragmentation of the attached bacteria was apparent (Fig. 3B). Within 90 min most of the bacteria appeared to be fragmented and deformed (Fig. 3C). In contrast, with chitosan alone the numbers of bacteria attached to the polymer appeared to be less than the numbers attached to the composite (Fig. 3D).

CLSM showed that GFP-expressing recombinant *E. coli* were attached all over the composite particles. CLSM micrographs of *E. coli* cells treated with the composite for 1 h showed considerable numbers of lysed bacteria with some extracellular fluorescence and fragmentation of cells (Fig. 4).

The fluorescence intensities of the GFP released into the medium by untreated, chitosan treated and composite treated bacterial cultures are shown in Fig. 5. The fluorescence spectroscopic studies showed that the amount of GFP released into the medium was much higher for both the composite and chitosan treated *E. coli* cells compared to the amount released by untreated cells (Fig. 5). The release of GFP into the medium,

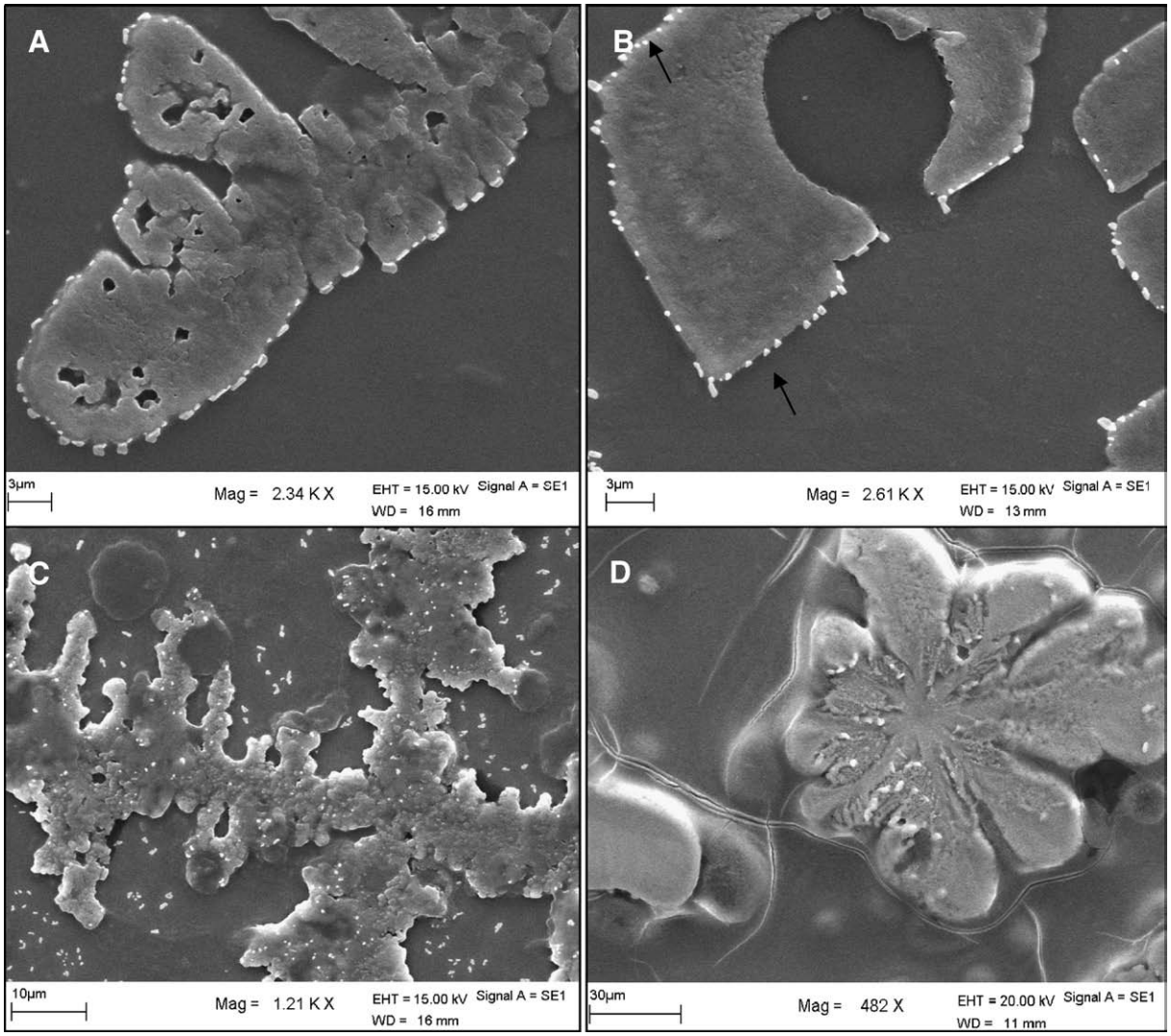

Fig. 3. SEM micrograph of *E. coli* cells after A. 30 min, B. 60 min, C. 90 min of treatment with chitosan–AgNPs composite material and D. after 90 min of treatment with chitosan alone.

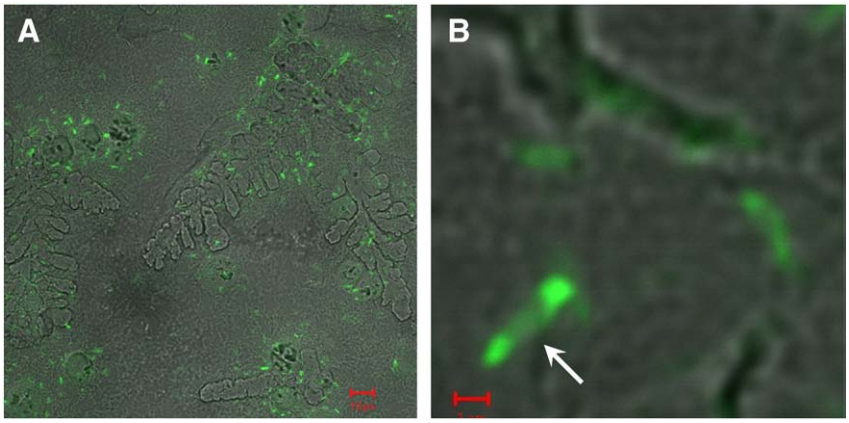

Fig. 4. A. CLSM micrograph of GFP-expressing *E. coli* after a 1 h incubation with chitosan–AgNPs composite. B. Fragmentation of bacteria after composite treatment.

which indicated the degree of membrane destabilization, was faster in the composite treated sample than in the chitosan treated sample.

Native PAGE showed that there were quantitative differences in protein profiles for untreated and composite treated samples. The growth of *E. coli* was responsible for the progressive increase in the band intensities in untreated samples. The band positions in the composite treated samples were similar to those in untreated samples at different times. In contrast, the band intensities in treated samples were less than those in untreated samples. Under UV trans-illumination the differences between untreated and treated samples with respect to GFP fluorescence were again apparent.

4. Discussion

Chitosan has strong affinity towards metal ions because of the presence of numerous amine and hydroxyl groups (Varma et al., 2004). Under alkaline condition chitosan can reduce Ag^{+} ions to AgNPs (Murugadoss et al., 2008). The product NPs become attached to the polymer, thus providing a single-step synthesis and stabilization of AgNPs. The AgNPs produced by this method were small and spherical.

Reported MBCs for chitosan against *E. coli* range from 0.0075–1.0% (w/v) (Rabea et al., 2003). Variations in the bactericidal efficiency of chitosan can be ascribed to the fact that antimicrobial activity of chitosan depends on a number of factors such as the molecular weight and degree of deacetylation of a chitosan preparation; the viscosity, ionic strength, pH and presence of metallic ions in the medium; and the temperature (Liu et al., 2006; No et al., 2002; Chung et al., 2003). The concentration of chitosan in composite preparations that completely inhibited the growth of *E. coli* was 0.012%. This value is comparable to corresponding values in other reports as the conditions of use in our study were generally much milder than those used in other studies. Considering the concentrations of the composite, the amount of AgNPs present in it, and the MIC and MBC values obtained, the corresponding AgNPs concentrations in the composite were 2.2 μg ml^{-1} and 2.6 μg ml^{-1}, respectively. Thus the MIC and MBC for AgNPs are much less than those previously reported for AgNPs (Gogoi et al., 2006). Also it is apparent that chitosan alone was less efficient than the composite for inhibiting growth of *E. coli*. The NPs synthesized in this study were less than 5 nm in diameter whereas the particles used in previous studies were about or less than 10 nm in diameter (Gogoi et al., 2006). Smaller NPs are known to be more reactive and hence could be more efficient in their antimicrobial activities (Gogoi et al., 2006). Also, chitosan itself is known to have strong antibacterial properties. The present investigation therefore indicates that the composite was more efficient than either AgNPs or chitosan alone for inactivating bacteria, possibly due to synergistic effect of both the AgNPs and chitosan in the composite.

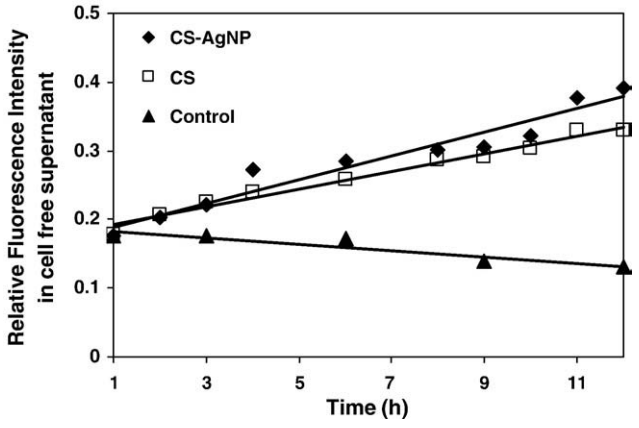

Fig. 5. Ratio of GFP fluorescence intensity in cell free supernatant to that of the total bacterial culture for 360 μg ml^{-1} of chitosan–AgNPs treated, 0.036% chitosan treated and control sample.

The outer membrane (OM) of Gram negative bacteria such as *E. coli* consists of lipopolysaccharides (LPS) containing phosphate and pyrophosphate groups which render the cell surface negatively charged (Prescott et al., 2002). As chitosan is a cationic polymer, it can attach to the *E. coli* cell wall by electrostatic interaction.

Below pH 6.5 chitosan can interact with the bacterial cell wall to destabilize it and alter cell permeability (Helander et al., 2001, 1998, 1997; Rabea et al., 2003). This process is probably enhanced by the binding of AgNPs to thiol-containing proteins present in the cell wall, with some of the AgNPs penetrating the cell wall to compromise permeability (Feng et al., 2000; Morones et al., 2005; Gogoi et al., 2006). This can lead to leakage of proteins and other intracellular constituents, and inactivation of the organisms (Helander et al., 2001; Rabea et al., 2003). The extracellular fluorescence observed under CLSM would be due to protein leakage. The progressive increase in GFP fluorescence in cell free supernatants confirmed the destabilization of the bacterial cell wall by the composite.

It has been proposed that AgNPs could interact with the sulfur containing intracellular proteins in bacteria (Feng et al., 2000; Morones et al., 2005). However, recent study has shown that AgNPs have no effects on the proteins of GFP-expressing *E. coli*. The present study showed that there was no apparent effect of the composite on the intracellular proteins in GFP-expressing *E. coli*.

Our work shows the promise of combining nanotechnology and biotechnology to develop antimicrobial agents with potential applications in food-related fields.

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References

Chung, Y.-C., Wang, H.-L., Chen, Y.-M., Li, S.-L., 2003. Effect of abiotic factors on the antibacterial activity of chitosan against waterborne pathogens. Bioresource Technology 88, 179–184.

Feng, Q.L., Wu, J., Chen, G.Q., Cui, F.Z., Kim, T.N., Kim, J.O., 2000. A mechanistic study of the antibacterial effect of silver ions on *Escherichia coli* and *Staphylococcus aureus*. Journal of Biomedical Materials Research 52, 662–668.

Gogoi, S.K., Gopinath, P., Paul, A., Ramesh, A., Ghosh, S.S., Chattopadhyay, A., 2006. Green fluorescent protein-expressing *Escherichia coli* as a model system for investigating the antimicrobial activities of silver nanoparticles. Langmuir 22, 9322–9328.

Helander, I.M., Alakomi, H.-L., Latva-Kala, K., Koski, P., 1997. Polyethyleneimine is an effective permeabilizer of Gram-negative bacteria. Microbiology 143, 3193–3199.

Helander, I.M., Latva-Kala, K., Lounatmaa, K., 1998. Permeabilizing action of polyethyleneimine on *Salmonella typhimurium* involves disruption of the outer membrane and interactions with lipopolysaccharide. Microbiology 144, 385–390.

Helander, I.M., Nurmiaho-Lassila, E.-L., Ahvenainen, R., Rhoades, J., Roller, S., 2001. Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria. International Journal of Food Microbiology 71, 235–244.

Liu, N., Chen, X.-G., Park, H.-J., Liu, C.-G., Liu, C.-S., Meng, X.-H., Yu, L.-J., 2006. Effect of MW and concentration of chitosan on antibacterial activity of *Escherichia coli*. Carbohydrate Polymers 64, 60–65.

Mcdonnell, G., Russell, A.D., 1999. Antiseptics and disinfectants: activity, action, and resistance. Clinical Microbiology Reviews 12, 147–179.

Morones, J.R., Elechiguerra, J.L., Camacho, A., Holt, K., Kouri, J.B., Ramírez, J.T., Yacaman, M.J., 2005. The bactericidal effect of silver nanoparticles. Nanotechnology 16, 2346–2353.

Murugadoss, A., Chattopadhyay, A., 2008. A 'green' chitosan–silver nanoparticle composite as a heterogeneous as well as micro-heterogeneous catalyst. Nanotechnology 19, 015603/1–015603/9.

No, H.K., Park, N.Y., Lee, S.H., Meyers, S.P., 2002. Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. International Journal of Food Microbiology 74, 65–72.

Prescott, L.M., Harley, J.P., Klein, D.A., 2002. Microbiology, 5th edition. The McGraw-Hill Companies, New York, pp. 46–61.

Rabea, E.I., Badawy, M.E.-T., Stevens, C.V., Smagghe, G., Steurbaut, W., 2003. Chitosan as antimicrobial agent: applications and mode of action. Biomacromolecules 4, 1457–1465.

Rhim, J.-W., Hong, S.-I., Park, H.-M., Ng, P.K.W., 2006. Preparation and characterization of chitosan-based nanocomposite films with antimicrobial activity. Journal of Agricultural and Food Chemistry 54, 5814–5822.

Varma, A.J., Deshpande, S.V., Kennedy, J.F., 2004. Metal complexation by chitosan and its derivatives: a review. Carbohydrate Polymers 55, 77–93.

Wang, X., Du, Y., Liu, H., 2004. Preparation, characterization and antimicrobial activity of chitosan–Zn complex. Carbohydrate Polymers 56, 21–26.

Wu, L.-Q., Gadre, A.P., Yi, H., Kastantin, M.J., Rubloff, G.W., Bentley, W.E., Payne, G.F., Ghodssi, R., 2002. Voltage-dependent assembly of the polysaccharide chitosan onto an electrode surface. Langmuir 18, 8620–8625.

Yi, Y., Wang, Y., Liu, H., 2003. Preparation of new crosslinked chitosan with crown ether and their adsorption for silver ion for antibacterial activities. Carbohydrate Polymers 53, 425–430.