Effect of chitosan molecular weight as micro and nanoparticles on antibacterial activity against some soft rot pathogenic bacteria

Ali Mohammadi a, Maryam Hashemi b,⁎, Seyed Masoud Hosseini c

a Department of Biology, Faculty of Sciences, Alzahra University, Tehran, Iran
b Microbial Biotechnology Department, Agricultural Biotechnology Research Institute of Iran (ABRII), Agricultural Research Education and Extension Organization (AREEO), Karaj, Iran
c Department of Microbiology, Faculty of Biological Sciences, Shahid Beheshti University, Evin, Tehran, Iran

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ABSTRACT

This study evaluates the in vitro antibacterial activity of three molecular weights of chitosan in the form of micro (CS) as well as nanoparticles (CSNPs). CSNPs were prepared using low (LWC), medium molecular weight (MWC) and middle-viscous crab shells chitosan (MVC). The antibacterial activities were evaluated through determination of IC50, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against Pseudomonas fluorescens, Erwinia carotovora and Escherichia coli. Experimental results showed that the antibacterial activity was significantly enhanced in the CSNPs comparison to CS microparticles, especially so for MVC nanoparticles (MVCN) in that the MIC values against all tested bacteria were ca. 50% those of the microparticles. Moreover, MWC and LWC nanoparticles showed lower MIC values compared with related microparticles only for P. fluorescens and E. coli, respectively. In addition, atomic force microscopy (AFM) of CSNPs-treated cultures of selected bacteria species revealed structural changes in the bacterial cell wall. The present study indicates that the antibacterial activity of CS varied depending on the molecular weight of CS micro and nanoparticles forms as well as on the particular bacterium. The selection of CS’s molecular weight could be thought to be more dependent on its application.

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1. Introduction

Chitosan (CS) is a natural cationic polysaccharide composed of β-(1→4) 2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D-glucopyranose and traditionally obtained from crustacean shells (crabs, shrimps and crayfish) using either chemical or microbiological procedures (Chung, Yeh, & Tsai, 2011; de Oliveira et al., 2014). Due to its unique biological characteristics, including biocompatibility, biodegradability and nontoxicity, many applications have been found either alone or blended with other natural polymers (alginites, starch, gelatin) in the food, agriculture, textile, water treatment, cosmetics and other industries (Kammoun, Haddar, Kalil, Dammak, & Sayari, 2013). Since CSs are active against foodborne pathogens, spoilage bacteria, pathogenic viruses and fungi (Hernández-Lauzardo, Velázquez-delValle, & Guerra-Sánchez, 2011; Kanatt, Chander, & Sharma, 2008), they have antibacterial activity already in the food matrix. Accordingly, they are added as an antibacterial compound to foods, such as fruits (cucumber, strawberry, grape, citrus, and tomato), fruit juices (orange, etc.), cereals, dairy (milk), eggs, seafood products and meat products (poultry, beef, bacon, pork, etc.) (Kanatt et al., 2008; Mohammadi, Hashemi, & Hosseini, 2015b; Mohammadi, Hashemi, & Hosseini, 2016).

The antibacterial activity of CS is exerted in various physical forms such as powders, solutions, suspensions, microspheres, films and coatings (Muzzarelli et al., 2012). In addition to tremendous advancements in cellular and molecular biological technologies and detecting methods, nanotechnology emerged and began playing an extraordinary role, carrying the potential to extend antimicrobial treatment to the atomic level (Kong, Chen, Xing, & Park, 2010). Nanoparticles display unique physical and chemical features because of effects such as the quantum size effect, mini size effect, surface effect and macro-quantum tunnel effect (Du, Niu, Xu, Xu, & Fan, 2009; Xu & Du, 2003). However, there is still some uncertainty on human health when nanoparticles are ingested or otherwise applied.
Chitosan tripolyphosphate nanoparticles (CSNPs) have been synthesized and mainly used as controlled-release drug carrier for gene transfer in artificial organs and for immune prophylaxis (Ting & Shen, 2005). In addition, it has shown its capacity for the encapsulation and delivery of polyphenolic compounds (Keawchoan & Yoksan, 2011; Mohammadi, Hashemi, & Hosseini, 2015a, 2015c; Rahaiee, Shojaosadati, Hashemi, Moini, & Razavi, 2015), hydrophilic and hydrophobic drugs (Trapani et al., 2011) and proteins (Avadi et al., 2010). In our previous study, CSNPs with a molecular weight of 684 kDa have shown its capacity for the loading and delivery of Cinnamomum zeylanicum and Zataria multiflora essential oils. Furthermore, the antifungal activity of EO when encapsulated by CSNPs under both in vitro and in vivo conditions were improved in comparison with unmodified oil (Mohammadi, 2015a, 2015c). Similarly, fruit coatings based on nanochitosan (50–110 nm) with and without copper loading improved the shelf-life and bioactive components of strawberry during cold storage (Eshghi et al., 2014). The unique character of nanoparticles for their small size and quantum size effect could make CSNPs exhibit superior antimicrobial activities.

Different physical states of CS, as a crucial factor influencing antimicrobial activity, have been considered but underestimated. CS’s water-solubility plays an important impact on its particular antimicrobial activities (Kong et al., 2010). In addition, molecular weight (MW) and molecular fraction of glucosamine units in the CS polymer chain (usually referred as the degree of deacetylation) influence CS solubility and interaction with the cell walls of target microorganisms (Tikhonov et al., 2006). Both parameters affect the antimicrobial activity of CS independently, though it has been suggested that the influence of the MW on the antimicrobial activity is greater than the influence of the deacetylation degree (DA) (Sekiguchi et al., 1993). Several studies have shown that the biological activity of CS depends significantly on its molecular weight (MW) (Badawy, Ahmed, & Rabea, 2006; Kim, Thomas, Lee, & Park, 2003; Liu et al., 2006; Tikhonov et al., 2006); however, most studies involve only one or two different MWs of CSs. Thus, information is lacking on the antibacterial activity of CSS with different MWs. Moreover, to our best knowledge, there is no comparative study on the antibacterial activities of CSNPs prepared from CS with different MWs against pathogenic bacteria. Therefore, the objective of the present research was to compare antibacterial activity of three CS with different MWs microparticle as well as their nanoparticles forms against three pathogenic bacteria including: Pseudomonas fluorescens, causing bacterial head rot of broccoli, Erwinia carotovora, causing soft mould disease and pathogenic Escherichia coli, causing bloody diarrhea. In addition, the morphological changes of tested bacteria treated with CSNPs were examined by AFM.

2. Materials and methods

2.1. Materials

CS was purchased from Sigma–Aldrich (St. Louis, MO, USA) as a powder material of different types: low molecular weight chitosan (LWC, MW = 70 kDa, DA = 75–85%), medium molecular weight chitosan (MWC, MW = 190 kDa, DA = 75–85%) and middle-viscous crab shell chitosan (MVC, MW = 684 kDa, DA = 75–85%) powder. Pentasodium tripolyphosphate, Na5P3O10 (TPP, M = 367.36 g/mol), acetic acid, CH3COOH (M = 60.05 g/mol) and sodium hydroxide (NaOH, M = 39.9971 g/mol) were purchased from Merck KGaA (Germany). All chemicals were of analytical grade and used as received. E. coli 0157:H7 ATCC. 25922, causing bloody diarrhea, P. fluorescens ATCC 17482, causing bacterial head rot of broccoli and E. carotovora PTCC No: 1675, causing soft mould disease for anti-bacterial study were purchased from the Iranian Research Organization for Science & Technology (IROST) on nutrient agar slants and kept at 4 °C. Subculturing was carried out each 14 days to preserve bacterial viability.

2.2. Methods

2.2.1. Preparation of CS solution

LWC, MWC and MVC solutions at concentrations of 3 g/L (0.3% w/v) were prepared by dissolving 300 mg of CS in 100 mL of 1% v/v acetic acid and mixed by stirring at ambient temperature (23–25 °C) overnight. The final pH of solutions was adjusted to 5.9 with 10% NaOH solution (Tsai & Su, 1999). Due to the poor solubility of CS, the mixtures were kept overnight at room temperature. The solutions were filtered through a sterilized 0.2 μm millipore syringe filter to remove any impurity before use. The CS stock solutions were diluted with 0.9% w/v NaCl solution to obtain the desired concentrations (0.15, 0.1, 0.075, 0.0375, 0.01875 and 0.009% (w/v)).

2.2.2. Preparation of CSNPs

CSNPs were prepared by ionic cross-linking of CS with TPP, which was previously described in the literature (Bhumkar & Pokharkar, 2006; Gan, Wang, Cochrane, & McCarron, 2005), with slight variation. Briefly, CS solution (0.3% w/v) was prepared by dissolving CS in aqueous acetic acid solution (1% v/v) at an ambient temperature overnight, followed by ultrasonication (MISONIX Inc. S–4000, USA) in an ice bath for 4 min at 60 W. A TPP solution (0.3% w/v) was prepared in distilled water. The final pH of CS and TPP solutions was adjusted to 4.6 and 5.6 using 1 N NaOH, respectively. CSNPs were spontaneously obtained by drop-wise adding TPP solution into the CS solution under constant stirring at room temperature for 60 min, followed by sonication for 14 min. The resultant suspensions were subjected to particle size analysis. Further, the prepared NPs were collected by centrifugation (TomyKongoYoCo.LTD. Suprema 25, JAPAN) at 27,000 × g for 14 min at 25 °C and washed with distilled water. The obtained particles were dispersed in distilled water and kept at 4 °C.

2.2.3. Characterization of CSNPs

The dynamic light scattering (DLS) measurements were performed with a particle size analyzer (StabiSizer 200, Particle Metrix GmbH, Germany). The measurements were performed at a temperature of 25 °C in triplicate. Samples were appropriately diluted with distilled water prior to measurement. The values were reported as mean ± standard deviation.

2.2.4. Nanoparticle yield determination

Each nanoparticle sample was centrifuged as described before and the deposit was lyophilized. Nanoparticle yield was calculated from the weight of the lyophilized nanoparticles (W2) and the sum of the initial dry weight of starting materials (W1) as using the following formula:

\[
\% \text{Percentage yield} = \frac{W_1}{W_2} \times 100
\] (1)

Results are reported as the mean of three measurements ± SD.

2.2.5. Determination of antibacterial activity

The inhibitory effects of micro and nano particles of chitosans with different MWs on the growth rate of indicator bacteria, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using the broth micro-dilution test recommended by the Clinical and Laboratory Standard Institute (CLSI, formerly NCCLS) (CLSI, 2012).

Cation-Adjusted-Müller-Hinton Broth (CAMHB) (pH 5.9) and
Mueller-Hinton Agar (MHA) were used as growth media. Serial doubling dilutions of CSs and their CSNPs in fresh CAMHB were performed in a 96-well microtiter plate (Nunc, Copenhagen, Denmark) over the range of 0.009%–0.15% w/v. Bacteria suspensions (after 16 h incubation) were inoculated to achieve a bacterial concentration of 1–2 × 10^5 CFU/ml in each well. The contents of microtiter plates were mixed with shaking for 10 min and incubated at 28 °C (P. fluorescens and E. carotovora) and 37 °C (E. coli) for 48 h. The optical density (OD) of each well was monitored by using a microplate reader (infinite M200 Pro, Tecan, Switzerland) at 600 nm (OD600) in 2-h intervals during the incubation time. After 24 h of incubation, the difference between optical densities of each sample was compared with a negative control (without CSs or CSNPs) then the MIC and MBC values were estimated. MIC values were determined as the lowest concentration of each CS which prevented visible turbidity in wells after 24 h. To evaluate MBC, a sample of 100 μl was transferred from each well without visible growth to a MH agar plate and incubated at 28 °C and 37 °C for an other 24 h. The MBC was read as the concentration of the tube without bacterial growth. Moreover, IC50 (concentration that produces a 50% inhibitory effect) values of antibacterial agents relative to controls were graphically obtained from the dose response curves based on measurement at six different concentrations.

2.2.6. Atomic force microscopy

The tested bacterial cells in the presence of the most promising treatments were imaged in air with a tapping mode atomic force microscope (Dimension Icon SPM, Bruker). Samples were prepared by applying 30 μL of bacterial suspension without treatment (control) or treated with CSNPs onto the surface of a piece of mica plate after 24 h for AFM observation, followed by air drying.

2.2.7. Statistical analysis

The results were analysed by a multifactor analysis of variance (ANOVA) and Tukey’s test with a 95% significance level using GraphPad Prism, version 5.02 (GraphPad Software, Inc., San Diego, CA, USA).

3. Results and discussion

3.1. Particle size and recovery yield

The recovery yield and mean particle size of different CSNPs with a constant amount of 0.3% w/v TPP are shown in Table 1. The mean particle size for three CSNPs was found to be statistically correlated with CS’s MW in which it increased when a higher MW was used (P < 0.05). As summarized in Table 1, LWC generally produced the smallest nanoparticles, followed by MWC and MVC. The results obtained are in agreement with those published recently (Yien, Zin, Sarwar, & Katas, 2012). They reported that the lower the CS’s MW, the smaller the particles derived from the CS and TPP reaction. It is due to the decreased viscosity which led to better solubility of CS in distilled water or acetic acid solution. Hence, more amino groups on CS would be protonated. This would lower the CS’s MW, the smaller the particles derived from the CS and TPP reaction. It is due to the decreased viscosity which led to lower the CS’s MW, the smaller the particles derived from the CS and TPP reaction. It is due to the decreased viscosity which led to lower the CS’s MW, the smaller the particles derived from the CS and TPP reaction. It is due to the decreased viscosity which led to.

These results indicate that the formation of NPs depended dramatically on the concentration of free amino groups, which increased the surface charge and zeta potential of the NPs and strengthened the electrostatic interactions between the CS and the TPP, helping to reduce the particle sizes (Yang, Wang, Huang, & Hon, 2010). Calvo, Remunan-Lopez, Vila-Jato, and Alonso (1997) pointed out that the concentrations of the CS and TPP solutions and the ratio of CS to TPP have important effects on nanochitosan preparation. The concentration of 0.3% (w/w) CS and TPP with weight ratios of 2.5:1 (CS: TPP) resulted in formation of NPs sized below 200 nm (polydispersity index < 1) as shown in Table 1. This finding is in agreement with Huang, Sheu, and Chao (2009) who suggested that the cross-linking was best when the LWCS/TPP mass ratio was approximately 5:2. When the TPP concentration was excessively low or high, it failed to react with the CS to form NPs. According to our results, the size of the formed LWCN, MWCN and MVCN was 60.00 ± 5.48, 78.50 ± 6.77 and 105.20 ± 8.58 nm, respectively. (Fig. 1)

Table 1

| Chitosan nanoparticles | Particle size (nm) (mean ± SD) | Recovery yield (%) |
|----------------------|-------------------------------|-------------------|
| LWCN                 | 60.00 ± 5.48                  | 27.6± 1.40%       |
| MWCN                 | 78.50 ± 6.77                  | 48.2± 3.20%       |
| MVCN                 | 105.20 ± 8.58                 | 88.6± 4.64%       |

![Fig. 1. Particle size distribution of: (A) Low molecular weight (LWCN); (B) medium molecular weight (MWCN); (C) middle-viscous crab shells (MVCN) chitosan nanoparticles. The size of LWCN, MWCN and MVCN range from 38.20 to 101.9 nm, 49 to 140.7 and 66.50–197.0 nm and the average size is about 60 nm, 78.50 nm and 105.2 nm, respectively.](image)
shows the particle size distribution curve of NPs made from three types of CS. A single peak occurred in the particle size distribution, indicating excellent uniformity.

The recovery yield of LWCN, MWCN and MVCN were 27.6 ± 1.40%, 48.2 ± 3.20% and 88.6 ± 4.64%, respectively. It is obvious that the highest recovery yield is achieved by MVCN.

3.2. Antibacterial assessment

The effects of different kinds of CS and related CSNPs on the growth of *P. fluorescens*, *E. carotovora* and *E. coli* after an incubation period of 24 h are presented in Table 2 and Figs. 2–4. The tested CSSs and CSNPs at concentrations ranging from 0.009 to 0.15% (w/v) significantly reduced the optical density (OD) values of bacteria when compared with the control (p < 0.05).

This inhibitory effect increased with increasing concentrations of CSSs and CSNPs (from 0.009 to 0.15% w/v) and was significantly related to the type of testing CS/CSNP and the species of bacteria (p < 0.05). It was very similar to other reports that the CS could inhibit the growth of bacteria at high concentration (Liu et al., 2006).

3.2.1. Anti *P. fluorescens* activity of CS with different molecular weight and related CSNPs

In case of *P. fluorescens*, over the range of 0.009–0.037% (w/v) for LWC, LWCN, MWCN and MVCN; 0.009–0.075% for MWC; and 0.018–0.075% for MVC, the difference between control and treatments was dramatic; the OD value in control increased over 1.5 but in samples supplemented with CSSs and CSNPs, it decreased to zero (Fig. 2). So the MIC values of all CS and CSNPs samples were 0.037% (w/v) (for all CSNPs and LWC) and 0.075% (w/v) (for MVC and MWC) against *P. fluorescens*. MWCN and MVCN with IC50 of 0.028% (w/v) were found to have better antibacterial activity against *P. fluorescens* compared with MWC and MVC. In contrast, MIC and IC50 of LWC were found to be similar between nanoparticle (LWCN) and its microparticle form. It was different from Yien et al. (2012) that reported the LWC has less antimicrobial activity against *C. albicans* compared with LWCN (Yien et al., 2012). Moreover, antibacterial activities of CSNPs against *P. fluorescens* were shown to be independent of CS’s MW as the IC50, MIC and MBC values of LWCN, MWCN and MVCN did not show significant difference. In contrast, a correlation between physical properties and antibacterial activities of CS microparticles has been statistically proven. The inhibitory activity of LWC, MWC and MVC against *P. fluorescens* decreased with the increasing MW as shown in Table 2. Among the evaluated microparticles, LWC with a MW of 70 kDa had the smallest particle size and showed the highest antibacterial effect against *P. fluorescens* with MIC of 0.037% (w/v). The results were little different from No, Young Park, Ho Lee, and Meyers (2002) that reported the growth of *P. fluorescens* was inhibited more effectively by CS of 476 kDa than by CS of 474, 224 or 28 kDa; however, further increase in MW reduced the antimicrobial activity observed for this bacterium.

3.2.2. Anti *E. carotovora* activity of CS with different molecular weight and related CSNPs

Against *E. carotovora*, when the concentration was higher than 0.018% (w/v), all CSNPs and related microparticles had shown their antibacterial activity obviously. The concentration of 0.037% (for LWC, LWCN, MWCN, MVCN and MWC) and 0.075% (for MVC) ensured complete inhibition of bacterial growth (Fig. 3). Therefore, the MIC of all treatments was 0.037% (w/v) (for all CSNPs, LWC and MWC) and 0.075% (w/v) (for MVC) against *E. carotovora*. Particle size was also found to have an influence on the inhibition of *E. carotovora* in the present study. In contrast to *P. fluorescens*, LWCN with an average diameter of 60 ± 5.48 nm and IC50 of 0.018% (w/v) had better inhibitory effects against *E. carotovora* compared to MWCN (78.50 ± 6.77 nm) and MVCN (105.2 ± 8.58 nm). In contrast to *P. fluorescens*, *E. carotovora* was found to be more susceptible to the inhibitory effects of MWC. For MWC, antibacterial activity was found to be similar between microparticle and nanoparticle forms.

3.2.3. Anti *E. coli* activity of CS with different molecular weight and related CSNPs

For *E. coli*, the OD values decreased with increasing concentrations of LWC, MWCN and MVCN (from 0.009 to 0.037% w/v), MWC

### Table 2

| Compound       | *P. fluorescens* % (w/v) | *E. carotovora* % (w/v) | *E. coli* % (w/v) |
|----------------|--------------------------|-------------------------|-----------------|
|                | MIC          | MBC         | IC50           | MIC           | MBC           | IC50           | MIC          | MBC           | IC50           |
| LWC            | 0.037        | 0.075       | 0.028          | 0.037         | 0.075         | 0.021          | 0.037        | 0.075         | 0.021          |
| LWCN           | 0.037        | 0.075       | 0.028          | 0.037         | 0.075         | 0.021          | 0.037        | 0.075         | 0.021          |
| MWC            | 0.018        | 0.075       | 0.043          | 0.018         | 0.075         | 0.043          | 0.018        | 0.075         | 0.043          |
| MWCN           | 0.018        | 0.075       | 0.043          | 0.018         | 0.075         | 0.043          | 0.018        | 0.075         | 0.043          |
| MVC            | 0.018        | 0.075       | 0.043          | 0.018         | 0.075         | 0.043          | 0.018        | 0.075         | 0.043          |
| MVCN           | 0.018        | 0.075       | 0.043          | 0.018         | 0.075         | 0.043          | 0.018        | 0.075         | 0.043          |

LWC = low molecular weight chitosan solution; MWC = medium molecular weight chitosan solution; MVC = middle-viscous chitosan solution; LWCN = low molecular weight chitosan nanoparticle suspension; MWCN = medium molecular weight chitosan nanoparticle suspension; MVCN = middle-viscous chitosan nanoparticle suspension.
The relationship between particle size and antibacterial activity was therefore studied against three different species of bacteria. In general, LWC were more effective in inhibiting bacterial growth than MWC and MVC. The latter two CSS required above 0.023% concentrations to produce a 50% inhibitory effect. These findings may differ from some other previous reported studies due to the differences in experimental conditions. Some studies reported increasing MW of CS lead to decreasing CS activity against E. coli (Zheng & Zhu, 2003), while in the others an increased activity for a high molecular weight CS (HWC) in comparison with LWC have been found (Kim et al., 2003). In contrast to the above mentioned results, no differences in HWC and LWC activities were found against E. coli (Jeon, Park, & Kim, 2001; Jia & Xu, 2001). For instance, Liu et al., (2006) noted that the antibacterial activity of LWC is higher than that of the HWC particles. But the CS with the middle MW (9.0 × 10^4 Da) could promote the growth of bacteria. Contrary to these results, Hwang, Kim, Yoon, and Pyun (1998) concluded that CS with MW about 30 KDa exhibited the highest bactericidal effect on E. coli from their investigation of MW range of 10–170 KDa (Hwang et al., 1998). Shimojoh, Masaki, Kurita, and Fukushima (1996) also found that CS of 220 KDa was most effective, whereas CS of 10 KDa was the least effective in their bacterial activities. However, the antimicrobial activity of CS of 70 KDa was better than that of 426 KDa for some bacteria, but for others the effectiveness was reversed. Badawy et al., (2006) examined the antimicrobial activity of 3.60 × 10^2, 6.11 × 10^2 and 9.53 × 10^2 KDa CSS against plant pathogenic bacteria Agrobacterium tumefaciens, Corynebacterium fascians, E. amylovora, E. carotovora, Pseudomonas solanacearum and Sarcina lutea. The results indicated that CSS of 6.11 × 10^2 and 9.53 × 10^2 kDa were more potent in bactericidal activity than CS of 3.60 × 10^2 kDa and CS of 9.53 × 10^2 kDa exhibited a good antibacterial potency especially against C. fascians with MIC of 500 mg/L. Also, No et al., (2002) found differences in MIC values with MWs of CS, ranging from 0.08% for CS of 746 or 470 KDa to 0.1% for CS of 28 or 224 kDa; however, further increase in MW (1106 or 1671 KDa) reduced the antimicrobial activity observed for E. coli. They reported that CS of 746 KDa appeared most effective against E. coli. Their reported MIC values are comparable to, or higher than, our current results. The discrepancies between data may result from the different DA and MW distributions of CS. Moreover, its antibacterial activity is reported to be affected by numerous intrinsic and extrinsic factors, such as microorganism species, presence or absence of metallic ions, pKa, pH, ionic strength, viscosity, concentration, physical state, hydrophilic/hydrophobic characteristic and chelating capacity of CS, etc. (Kong et al., 2010; Sanpui, Murugadoss, Prasad, Ghosh, & Chattopadhyay, 2008; Tsai, Zhang, & Shieh, 2004).

3.2.4. Differences in antibacterial efficacy between bacterial species/strains

Our present study revealed that the effectiveness of CS did not depend solely on the CS formulation, but also on the type of bacterium. The relationship between particle size and antibacterial activity was therefore studied against three different species of bacteria. In general, LWC were more effective in inhibiting bacterial growth than MWC and MVC. The latter two CSS required above 0.023% concentrations to produce a 50% inhibitory effect. These findings may differ from some other previous reported studies due to the differences in experimental conditions. Some studies reported increasing MW of CS lead to decreasing CS activity against E. coli (Zheng & Zhu, 2003), while in the others an increased activity for a high molecular weight CS (HWC) in comparison with LWC have been found (Kim et al., 2003). In contrast to the above mentioned results, no differences in HWC and LWC activities were found against E. coli (Jeon, Park, & Kim, 2001; Jia & Xu, 2001). For instance, Liu et al., (2006) noted that the antibacterial activity of LWC is higher than that of the HWC particles. But the CS with the middle MW (9.0 × 10^4 Da) could promote the growth of bacteria. Contrary to these results, Hwang, Kim, Yoon, and Pyun (1998) concluded that CS with MW about 30 KDa exhibited the highest bactericidal effect on E. coli from their investigation of MW range of 10–170 KDa (Hwang et al., 1998). Shimojoh, Masaki, Kurita, and Fukushima (1996) also found that CS of 220 KDa was most effective, whereas CS of 10 KDa was the least effective in their bacterial activities. However, the antimicrobial activity of CS of 70 KDa was better than that of 426 KDa for some bacteria, but for others the effectiveness was reversed. Badawy et al., (2006) examined the antimicrobial activity of 3.60 × 10^2, 6.11 × 10^2 and 9.53 × 10^2 KDa CSS against plant pathogenic bacteria Agrobacterium tumefaciens, Corynebacterium fascians, E. amylovora, E. carotovora, Pseudomonas solanacearum and Sarcina lutea. The results indicated that CSS of 6.11 × 10^2 and 9.53 × 10^2 kDa were more potent in bactericidal activity than CS of 3.60 × 10^2 kDa and CS of 9.53 × 10^2 kDa exhibited a good antibacterial potency especially against C. fascians with MIC of 500 mg/L. Also, No et al., (2002) found differences in MIC values with MWs of CS, ranging from 0.08% for CS of 746 or 470 KDa to 0.1% for CS of 28 or 224 kDa; however, further increase in MW (1106 or 1671 KDa) reduced the antimicrobial activity observed for E. coli. They reported that CS of 746 KDa appeared most effective against E. coli. Their reported MIC values are comparable to, or higher than, our current results. The discrepancies between data may result from the different DA and MW distributions of CS. Moreover, its antibacterial activity is reported to be affected by numerous intrinsic and extrinsic factors, such as microorganism species, presence or absence of metallic ions, pKa, pH, ionic strength, viscosity, concentration, physical state, hydrophilic/hydrophobic characteristic and chelating capacity of CS, etc. (Kong et al., 2010; Sanpui, Murugadoss, Prasad, Ghosh, & Chattopadhyay, 2008; Tsai, Zhang, & Shieh, 2004).
antimicrobial activity is. However, it is worthwhile to note that all the CSNPs were more active than CS microparticle forms and exhibited a good bactericidal activity against the tested bacteria. Based on the results obtained, MVCN showed lower MIC compared with MVC against all bacterial cultures tested. Moreover, MIC values of MWCN and LWCN were lower compared with MWC and LWC only for *P. fluorescens* and *E. coli*, respectively. This finding was in agreement with other study which demonstrated that CSNPs exhibited higher antimicrobial activity due to their special characters of the NPs such as small and compact particle as well as high surface charge (Qi, Xu, Jiang, Hu, & Zou, 2004). This could be explained by the fact that the negatively charged surface of the bacterial cell is the target site of polycation (Singh, Vesentini, Singh, & Daniel, 2008). Therefore, the CSNPs with polycations structure

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**Fig. 5.** AFM images of *P. fluorescens*, before (A) and after treatment with Low molecular weight (LWCN) (B), medium molecular weight (MWCN) (C) and middle-viscous crab shells (MVCN) chitosan nanoparticles (D); *E. carotovora*, before (E) and after treatment with Low molecular weight (LWCN) (F), medium molecular weight (MWCN) (G) and middle-viscous crab shells (MVCN) chitosan nanoparticles (H); *E. coli*, before (I) and after treatment with Low molecular weight (LWCN) (J), medium molecular weight (MWCN) (K) and middle-viscous crab shells (MVCN) chitosan nanoparticles (L).
and high surface charge density will interact more effectively with the bacteria compared with microparticle forms (Qi et al., 2004). Furthermore, CSNPs provide higher affinity with bacteria cells for a quantum-size effect. Because of the larger surface area of the nanosized CSNPs, NPs could be tightly adsorbed onto the surface of the bacteria cells so as to disrupt the normal functions of the membrane, e.g. by promoting the leakage of intracellular components or by inhibiting the transport of nutrients into cells, thus killing the bacteria cells (Devlieghere, Vermeulen, & Debevere, 2004; Qi et al., 2004). In the study of Ma and Lim (2003) cellular uptake of CSNPs into cells was higher than that of CS microparticle forms as the bulk CS molecules were located extracellularly. This suggested that CSNPs might be able to diffuse into bacterial cell and hence disrupt the synthesis of DNA as well as RNA. This could
explain a better antibacterial efficacy of CSNPs compared to its microparticle form. The findings of this study may differ from some of other previous reports due to the differences in experimental conditions. Further investigation on different species of bacteria is being carried out because type of bacteria is also affecting antibacterial activity of CS.

3.3. Atomic force microscopy (AFM)

On the basis of the obtained results that the antibacterial activity of CS microparticle forms was significantly enhanced by the CSNPs, only the morphology of CSNPs-treated bacteria were examined by AFM. When the bacteria were treated with 0.075% (w/v) of LWG, MWG and MCV nanoparticles for 24 h, the cells were disrupted to a considerable degree with the leakage of cytosolic components, membrane sloughing, and breaching (Fig. 5). The cocci or the spheroid cell shape morphological alterations is evident from the AFM images of the untreated cells, as shown in Fig. 5A, E and I. AFM image analysis revealed that the surface ultrastructures of all strains treated with LWGNC, MWGNC and MCVNC are completely different. The healthy bacteria cells are distinguishable from the CSNPs exposed cells. The untreated P. aeruginosa and E. carotovora cells have a relatively smooth surface without pores or any ruptures in comparison with the grooves on the CSNPs-treated cells. Grooves or depressions or holes distributed on every cell surface demonstrate the shape alterations induced by CSNPs.

The microtitre plate assay results conform to the atomic force microscopy image analysis. This is the first visual demonstration of the effect of CSNPs on pathogenic bacterial cells. The effects of various CSNPs on the morphometric and the surface structures of tested bacterial cells revealed that CSNP forms could be effective as antibiotic agents for treating infections caused by bacteria. Further research on in vivo studies on the efficacy of CSs and CSNPs in treating pathogenic bacteria is recommended.

4. Conclusions

A positive correlation between particle size of CSNPs and molecular weight of CS was statistically proven, in which it increased when a higher MW was used. The reduction in the OD of cell suspension depends on the type of CS microparticles, CSNPs and the species of bacteria. It is difficult to determine what the most optimal MW for the maximal antimicrobial activity is. The selection of MW of CS could be thought to be more dependent on its application. However, MCVN exhibit higher antibacterial activity than MVD itself against all bacterial cultures tested. Moreover, MWGNC and LWGNC showed lower MIC and MBC values compared with related microparticles only for P. aeruginosa and E. coli, respectively. Their MIC values were less than 0.037% (w/v). AFM of CSNPs-treated cultures of tested bacteria revealed that the antibacterial action was probably via membrane disruption and leakage of cellular protein so as to kill the bacteria cells due to the change of membrane permeability. It is anticipated that CSNPs could be applied broadly as antimicrobial agents in medicine and food science for their high antibacterial activity and acceptable biocompatibilities.

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