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Physicochemical Effects of Chitosan-Tripolyphosphate Nanoparticles on Antibacterial Activity against Gram-positive and Gram-negative Bacteria

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Recently, increasing attention has been focused to develop nanoparticles based on chitosan. Chitosan has been shown to exhibit a high antibacterial activity against Gram-positive and also Gram-negative bacteria. The study therefore, aims to determine antimicrobial activity of chitosan nanoparticles *in vitro* on *S. aureus* and *E. coli*. Nanoparticles were prepared using ionic gelation method. Antibacterial activity of these nanoparticles was then determined by measuring their Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). MIC and MBC values were in the range of 0.24-31.25 and 1.95-125 μg mL^{-1}, respectively depending on the particle size and suspending mediums. Smaller nanoparticles (±400 nm) showed higher antibacterial activity in comparison to larger ones (±700 nm). Smaller particles have larger surface areas which would be in contact with the bacteria, hence gave better inhibition. In conclusion, chitosan demonstrated its antibacterial activity as nanoparticle form and the activity was mainly influenced by its particle size.

Key words: Ionic gelation, antimicrobial, chitosan, nanoparticles, particle size

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INTRODUCTION

Chitosan has been used extensively not only in biomedical fields but also in development of drug delivery systems (Avadia *et al*., 2004; Hayashi, 1994). In fact, the use of chitosan in drug delivery systems has become increasingly important due to its advantages such as being non toxic, biodegradable and biocompatible with human body. Furthermore, it has been determined that chitosan has antimicrobial effects against both Gram-negative and Gram-positive bacteria which has attracted considerable interest to its potential in pharmaceutical fields. Many reports have been published on the antimicrobial activity of chitosan which is not only limit to solutions (Zheng and Zhu, 2003) but also, other forms such as complexes (Wang *et al*., 2004) and nanoparticles (Qi *et al*., 2004; Sadeghi *et al*., 2008). These investigations have also shown that inhibitory effects of chitosan against bacteria depended on its molecular weight (No *et al*., 2002), degree of deacetylation (Jeon *et al*., 2001), pH (Holappa *et al*., 2006), temperature (Tsai and Su, 1999), concentration (Wang *et al*., 2004) and water solubility of chitosan (Qin *et al*., 2006). However, these studies were performed in different conditions and some results obtained were conflicting. Recently, many researches on antibacterial effect of chitosan nanoparticles have also been reported. However, information on the correlation between physical characteristics of these nanoparticles and their antibacterial effects is still lacking. Therefore, the aim of this present study is to investigate the relationship between the physical characteristics of chitosan nanoparticles with respect to their particle size and surface charge against their antibacterial activity. In addition, the antibacterial effects of these nanoparticles were compared to chitosan in its free polymer form using various suspending medium.

MATERIALS AND METHODS

Materials: Chitosan; high molecular weight (HMW; molecular weight between 50-90 kDa, degree of deacetylation between 75-85%) and low molecular weight (LMW; molecular weight = 310 kDa with 85% degree of deacetylation) were purchased from Sigma, Germany. Penta-sodium triphosphate (TPP) was obtained from Merck, Germany. *Staphylococcus aureus* ATCC 27661 and *Escherichia coli* NCTC 10964 were obtained from ATCC. Muller Hinton Agar (MHA) and Muller Hinton Broth (MHB) were purchased from Merck, Germany. All other chemicals were of pharmaceutical and analytical grade and were used as received.

Preparation of chitosan-TPP nanoparticles: The ionic gelation method was used to prepare nanoparticles from chitosan. A stock solution of chitosan (1% w/v) was prepared by dissolving chitosan in 2% v/v acetic acid. A series of chitosan concentrations was prepared by diluting a stock solution with distilled water to produce 0.1, 0.2 and 0.3% w/v chitosan solutions. To prepare nanoparticles, 7.2 mL TPP solution (0.1% w/v in distilled water) was added into 18 mL chitosan solution drop-wise under magnetic stirring at 700 rpm and at room temperature. After 1 h incubation at room temperature, samples were centrifuged twice at 45 000 rpm for 30 min at 10°C (Beckman Ultracentrifuge, USA). After centrifugation, pellets of nanoparticles were resuspended in distilled water for further analysis.

Characterisation of chitosan-TPP nanoparticles: Particle size and surface charge (zeta potential) of freshly prepared nanoparticles and nanoparticles after centrifugation were measured using a Malvern Zetasizer (3000 ZS, UK). Each sample was measured in triplicates and the values are presented as the Mean±Standard deviation (SD).

Determination of MIC: The minimum inhibitory concentration (MIC) of chitosan and its nanoparticles was determined by turbidimetric method. To the first tube, 100 μL of chitosan or nanoparticles (1 mg mL^{-1}) in acetic acid or distilled water was added into 100 μL of MHB. After mixing, 100 μL of the mixture was transferred to the second tube. Then, 100 μL from the second tube was transferred to the third and the same transformations were repeated for the following tubes. Hence, each tube would contain a solution with half of the concentration of the previous one. After dilution, the tubes were inoculated under aseptic condition with 100 μL of *E. coli* or *S. Aureus* inoculum suspensions ($1\text{-}2\times10^5$ CFU mL^{-1}) and therefore, the final volume for each tube was 200 μL. After mixing, the tubes were incubated at 37°C for 24 h. MIC was read after 24 h of incubation at 37°C equivalent to the concentration of the tube without visible growth. The lowest concentration of chitosan and chitosan nanoparticles that inhibited the growth of bacteria was considered as the minimum inhibitory concentration or MIC. The control tubes contained antibiotic broth and acetic acid, without bacteria or chitosan. Ampicillin was used as antibacterial standard. To study the effect of distilled water as a suspending medium for nanoparticles on antibacterial activity, the same treatment was performed as above, except acetic acid was replaced with distilled water.

Determination of MBC: The minimum bactericidal concentration (MBC) was determined by assaying the live organisms in those tubes from the MIC test that showed

no growth. A volume of 20 μL from each of those tubes without visible growth was transferred to a MHA plate and signs of growth after 24 h incubation at 37°C were noted. The MBC was read as the concentration of the tube without bacterial growth. The test was performed triplicate for each bacterium. Ampicillin was used as antibacterial standard.

RESULTS

Particle size: The results obtained suggested that the size and surface charge of nanoparticles were influenced by chitosan concentration and molecular weight used. An increase of particle size was observed with the increased of chitosan concentration used during the preparation of nanoparticles. The size of particle increased from 100±0.7 to 356±17 nm when LMW chitosan concentration was increased from 0.1 to 0.4% w/v (Table 1). A similar trend was also observed for HMW chitosan in which particle size greatly increased from 198±5.6 to 2148±78 nm when the concentration increased from 0.2 to 0.4% w/v (Table 2). In general, chitosan with lower molecular weight (LMW) produced smaller particle size than chitosan with a higher molecular weight (HMW). Furthermore, LMW chitosan produced a better colloidal system with a narrow particle size distribution than that of HMW chitosan.

Stability of the chitosan nanoparticle system against centrifugation was also influenced by the concentration and molecular weight of chitosan used in the preparation. For the LMW chitosan, particle size after centrifugation increased up to 0.7 and was 1.3 times greater for the concentrations at 0.3 and 0.2% w/v. On the other hand, the size of nanoparticles made from 0.4% w/v of chitosan remained the same before and after centrifugation. However, nanoparticles prepared from 0.1% w/v could not be measured due to the occurrence of phase separation after centrifugation (Table 1). In contrary, particle size of nanoparticles prepared from HMW chitosan at all studied concentrations reduced significantly after centrifugation except for concentration of 0.2% w/v where the particle size increased by 63% from 198.2±5.6 to 323.2±16 nm (Table 2).

Table 1: Particle size and polydispersity index of chitosan nanoparticles before and after centrifugation for LMW chitosan (n = 3)

Chitosan concentration (% w/v)	Centrifugation	Particle size (nm)	Polydispersity index
0.1	Before	100.8±0.7	0.290±0.01
	After	N/A	N/A
0.2	Before	153.9±2.3	0.441±0.02
	After	390.2±65	0.645±0.15
0.3	Before	223.5±8.0	0.631±0.13
	After	381.6±11	0.666±0.08
0.4	Before	356.4±37	0.581±0.16
	After	356.6±17	0.463±0.05

Particle surface charge: The particle surface charge of nanoparticles obtained from ionic gelation method increased with the increased concentration of chitosan (0.1-0.4% w/v) for both types; LMW and HMW chitosan. The results obtained also suggested that HMW chitosan produced a higher value of particle surface charge compared to LMW chitosan with an exception for 0.2% w/v chitosan. However, the values decreased after centrifugation for all the samples irrespectively of their molecular weight (Table 3).

Antibacterial effects

Comparison of antibacterial effect between free and nanoparticle forms: Table 4 shows the antibacterial effect of chitosan in its free form and as nanoparticles form. According to the results, chitosan solution has less inhibitory effects than the nanoparticles although the nanoparticles were re-suspended in the same medium as chitosan which is acetic acid. Acetic acid was studied because it has been used in the preparation of chitosan nanoparticles to dissolve chitosan. A concentration of 0.02% v/v of acetic acid was expected as a minimum concentration after taking into consideration a series of dilution during nanoparticles preparation and therefore chosen for the antibacterial studies. A similar inhibition effect was observed for both types of bacteria; *S. aureus* and *E. coli*.

Effect of particle size and surface charge: The results showed that the inhibitory effect of chitosan nanoparticles against *S. aureus* and *E. coli* was influenced by particle size. A greater inhibitory effect was

Table 2: Particle size and polydispersity index of chitosan nanoparticles before and after centrifugation for HMW chitosan (n = 3)

Chitosan concentration (% w/v)	Centrifugation	Particle size (nm)	Polydispersity index
0.1	Before	N/A	N/A
	After	N/A	N/A
0.2	Before	198.2±5.6	0.509±0.02
	After	323.2±16	0.576±0.12
0.3	Before	871.8±15	1.000
	After	727.3±128	0.990±0.02
0.4	Before	2148±78	1.000
	After	1061.63±17	0.963±0.07

Table 3: Particle surface charge of chitosan nanoparticles before and after centrifugation (n = 3)

Chitosan concentration (% w/v)	Centrifugation	Particle surface (mV)	
		LMW	HMW
0.1	Before	25.5±0.01	N/A
	After	N/A	N/A
0.2	Before	47.0±1.6	37.5±0.7
	After	36.1±0.6	32.9±4.1
0.3	Before	52.0±1.1	63.6±3.6
	After	38.1±1.1	59.4±0.9
0.4	Before	54.0±1.6	73.1±2.3
	After	43.6±0.05	67.1±1.8

Table 4: MIC (μg mL^{-1}) and MBC (μg mL^{-1}) of chitosan solution and chitosan nanoparticles against *S. aureus* and *E. coli* in acetic acid 0.02% v/v

	Chitosan solution				LMW: *Chitosan concentration						HMW: *Chitosan concentration							
					0.2% w/v		0.3% w/v		0.4% w/v		0.2% w/v		0.3% w/v		0.4% w/v			
	LMW		HMW		390 nm		381 nm		357 nm		323 nm		727 nm		1061 nm		Control ampicillin	
Particle size	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
S. aureus	31.3	250	93.8	375	0.98	7.81	0.98	7.81	0.98	7.81	0.98	7.81	3.91	15.6	15.6	62.5	0.5	0.5
E. coli	31.3	125	62.5	250	0.49	1.95	0.24	1.95	0.24	1.95	0.24	1.95	0.98	7.81	1.95	15.6	4	4

*Chitosan concentration used to prepare nanoparticles

Table 5: MIC (μg mL^{-1}) and MBC (μg mL^{-1}) of chitosan nanoparticles against *S. aureus* and *E. coli* in distilled water

	*Chitosan concentration													
	LMW						HMW							
	0.2% w/v 390 nm		0.3% w/v 381 nm		0.4%w/v 357 nm		0.2% w/v 323 nm		0.3% w/v 727 nm		0.4%w/v 1061 nm		Control ampicillin	
Particle size	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
S. aureus	1.95	15.6	1.95	15.6	1.95	15.6	1.95	15.6	7.81	31.3	31.3	125	0.5	0.5
E. coli	0.98	7.81	0.98	7.81	0.98	7.81	0.98	0.98	3.91	15.6	15.6	62.5	4	4

*Chitosan concentration used to prepare nanoparticles

observed with increased particle size of chitosan nanoparticles. The MIC and MBC values decreased from 0.98 to 15.6 μg mL^{-1} and 7.81 to 62.5 μg mL^{-1}, respectively when the size of nanoparticles increased from approximately 400 to 1000 nm (Table 4). Since the size of chitosan nanoparticles prepared by ionic gelation is affected by chitosan molecular weight, one can assume that antibacterial effects of chitosan nanoparticles would also indirectly be affected by molecular weight as well as concentration of chitosan used in the preparation. In general, a lower molecular weight (LMW) or concentration of chitosan produced smaller nanoparticles than higher molecular weight (HMW) chitosan, hence has a higher inhibitory effect against Gram-positive and Gram-negative bacteria growth. In contrast, there was no distinct antibacterial effects with respect to the different particle surface charge.

Effect of suspending medium: The effect of distilled water as a suspending medium for chitosan nanoparticles was also studied to investigate its antibacterial effect against Gram-positive and Gram-negative bacteria. The results indicated that the growth inhibitory effect against *S. aureus* and *E. coli* was greater in acetic acid than in distilled water (Table 5). Table 4 and 5 also show that inhibitory effects of chitosan nanoparticles which is higher against *E. coli* compared to *S. aureus*. MIC and MBC values for the nanoparticles in acetic acid increased 17-and 2-folds, respectively than the control ampicillin. These particles were in the size range between 330-380 nm irrespectively of chitosan molecular weight. However, in distilled water, all the particles in the above size range showed MIC values less than 4 μg mL^{-1} (ampicillin) but all their MBC values were well above the control except for the particles made from 0.2% w/v HMW chitosan. The nanoparticles made from 0.2% w/v HMW chitosan had both MIC and MBC values of 0.98 μg mL^{-1} which is less than the control (Table 5).

DISCUSSION

The ionic gelation method is a simple and mild method to prepare chitosan nanoparticles and was first introduced by Calvo *et al.* (1997). In this method, chitosan forms a gel spontaneously upon contact with multivalent polyanions such as TPP ions through the formation of inter- and intramolecular cross-linkage mediated by polyanions (Gan *et al.*, 2005). In this study, it was found that particle size of chitosan nanoparticles was influenced by chitosan concentration and molecular weight.

The comparative value of surface charge (zeta potential) of chitosan nanoparticles increased with the increasing concentration of chitosan. The increment was due to the increase number of positive charges which counteracted with negative charges of TPP ions, as the amount of TPP was fixed. The net positive charge of the particles is desirable to prevent particle aggregation and promote electrostatic interaction with the overall negative charge of microbial cells (Qi *et al.*, 2004). However, the positive values of these nanoparticles decreased after centrifugation. A possible reason was it might be due to the medium where the particles were suspended in. Before centrifugation, particles were suspended in acetic acid since chitosan would only dissolved in acetic acid. The pH of the nanoparticle system right after ionic gelation process was about 4.5 and the particle surface charge was in the range of +47-53 mV. But, after centrifugation, all the particles were re-suspended in

distilled water (pH about 6.5) and this was thought to be a reason for the reduction of particle surface charge, where the values fell into the range of +36-67 mV.

Antibacterial effect of chitosan has been reported due to a high density of amino groups which confers a positive charge to the polymer and attributed to the formation of polyelectrolyte complexes with the negative peptidoglycans of the bacterial cell wall. This interaction may in turn disrupt cell wall and lead to the inhibition of the bacterial growth (Sadeghi *et al*., 2008). In this study, the antibacterial effect of the polymer in solution was compared to its nanoparticle form. The results obtained indicated that chitosan nanoparticles had a higher antibacterial activity against *S. aureus* and *E. coli* than the free polymer. This finding contradicted the finding by Sadeghi *et al.* (2008) which demonstrated otherwise. According to the report, the effect is might be due to the decrease number of positive charge available for interaction with the bacterial cell wall since formation of nanoparticles by ionic gelation also involves interaction between chitosan amino groups and polyanions. This is supported by a lower particle surface charge of the nanoparticles (+15.9 mV) in comparison to the free polymer form (Sadeghi *et al*., 2008). In our case, the obtained nanoparticles had a high surface charge, mostly more than +30 mV which therefore, did not compromise in their ability to interact with the bacterial cell wall. In addition to that, a better inhibitory effect by the nanoparticles was thought to be due to the larger surface area of the nanoparticles available for the interaction with the bacterial cell wall. These nanoparticles could also be tightly adsorbed onto the surface of bacteria cell wall and consequently, this would disrupt the cell membrane and kill the bacteria (Qi *et al*., 2004).

This also coincides with our findings which demonstrated that the inhibitory effect against *S. aureus* and *E. coli* by nanoparticles increased with the decrease size of nanoparticles which may also relate to the surface area being in contact with the bacterial cell wall. The smaller the particle size, the greater is the surface area which could interact with bacterial cell wall. On the other hand, the data demonstrated that HMW chitosan had a lesser inhibitory effect than LMW in free form. These observations were in agreements with previous report by Liu *et al.* (2006). Higher molecular weight chitosan gave low inhibitory effects and this could be due to its compact arrangement which in turn limit its cationic groups to interact with the cell surface of bacteria. For nanoparticles however, no clear relationship was observed between antibacterial effects and the molecular weight of chitosan. Although, nanoparticles prepared from LMW produced greater inhibitory effects compared to HMW but the same effects was also observed for nanoparticles prepared from HMW but with a similar size to that of LMW nanoparticles. This therefore, suggested that particle size played a more important role in antibacterial activity than molecular weight or concentration used to prepare nanoparticles. As expected, a lower antibacterial activity of chitosan nanoparticles suspended in distilled water was observed than the one in acetic acid against both tested bacteria. This result coincides with the study done by Qi *et al.* (2004). Thus, it showed that the antibacterial activity of chitosan was affected by pH and higher activity was observed at lower pH values. This is due to protonation of free amino groups of chitosan in acidic condition and therefore, chitosan renders a positive charge at pH below 6.5 (Avadia *et al*., 2004).

All samples showed a higher antibacterial activity against *E. coli* than *S. aureus*. A higher activity can be attributed to a higher negative surface charge of bacteria Gram-negative (*E. coli*) than Gram positive (*S. aureus*) (Chung *et al*., 2004) which is also responsible in promoting the binding of polycationic groups of chitosan to anionic molecules at the bacteria cell wall surface (Jumaa *et al*., 2002). Interestingly, all nanoparticles with the particle size less than 400 nm showed a higher antibacterial activity than ampicillin against *E. coli*. This, therefore strongly suggested that the antibacterial activity of these nanoparticles is highly dependent on the particle size. However, more work is needed to confirm this hypothesis.

CONCLUSIONS

In summary, this study showed that chitosan nanoparticles could inhibit the growth of both *S. aureus* and *E. coli*. These nanoparticles also exhibited a higher antibacterial activity than free polymer form in solution and the control; ampicillin. The inhibitory effect of chitosan nanoparticles was found to be influenced mainly by their particle size besides other factors such as pH of suspending medium. Therefore, it is anticipated that chitosan nanoparticles have a great potential to be applied in pharmaceutical as antimicrobial agent.

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