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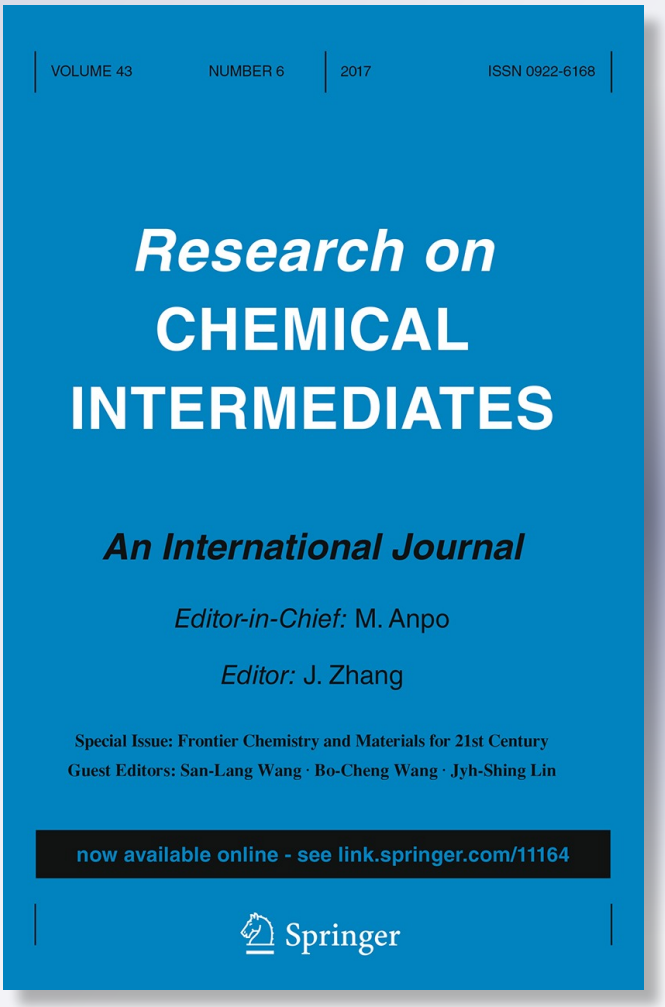

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Preparation of chitosan nanoparticles by TPP ionic gelation combined with spray drying, and the antibacterial activity of chitosan nanoparticles and a chitosan nanoparticle–amoxicillin complex

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Abstract Chitosan nanoparticles were prepared from chitosan with various molecular weights by tripolyphosphate (TPP) ionic gelation combined with a spray drying method. The morphologies and characteristics of chitosan nanoparticles were determined by TEM, FE-SEM and from their mean sizes and zeta potentials. The effect of chitosan molecular weight (130, 276, 760 and 1200 cPs) and size of spray dryer nozzle (4.0, 5.5 and 7.0 μm) on mean size, size distribution and zeta potential values of chitosan nanoparticles was investigated. The results showed that the mean size of chitosan nanoparticles was in the range of 166–1230 nm and the zeta potential value ranged from 34.9 to 59 mV, depending on the molecular weight of chitosan and size of the spray dryer nozzles. The lower the molecular weight of chitosan, the smaller the size of the chitosan nanoparticles and the higher the zeta potential. A test for the antibacterial activity of chitosan nanoparticles (only) and a chitosan nanoparticle–amoxicillin complex against *Streptococcus pneumoniae* was also conducted. The results indicated that a smaller chitosan nanoparticle and higher zeta potential showed higher antibacterial activity. The chitosan nanoparticle–amoxicillin complex resulted in improved antibacterial activity as compared to amoxicillin and chitosan nanopaticles alone. Using a chitosan nanoparticle–amoxicillin complex could reduce by three times the dosage of amoxicillin while still completely inhibiting *S. pneumoniae*.

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Keywords Chitosan nanoparticles · Antibacterial activity · Amoxicillin · *Streptococcus pneumoniae*

Introduction

Streptococcus pneumoniae is a Gram-positive, normal inhabitant of the human upper respiratory tract. The bacteria can cause pneumonia, usually of the lobar type, paranasal sinusitis and otitis media which are usually secondary to one of the former infections. It also causes osteomyelitis, septic arthritis, endocarditis, cellulitis and brain abscesses. *S. pneumonia* currently is one of the leading causes of invasive bacterial disease in children. Particularly, *S. pneumonia* is strongly resistant to antibiotics. Therefore, looking for the best way to control this bacterium is a very important obligation in the pharmaceutical field [1–3].

Chitosan is a natural polymer of beta 1,4-glucosamine and beta 1,4-*N*-acetyl glucosamine residues. Chitosan is a non-toxic, biocompatible, biodegradable and natural polycationic agent and has great potential for agricultural [4–7] and biomedical applications, such as a promising carrier for proteins, drug delivery, vaccine delivery and antitumor treatment [8–12].

Chitosan nanoparticles are nanosized, have a large surface area, a high zeta potential, and they can provide superior activities. Recently, chitosan nanoparticles have been studied and applied in agriculture; Sang et al. [13] applied chitosan nanoparticles for coffee seedlings in the green house to enhance the growth and resistance of diseases. In the biomedical field, chitosan nanoparticles have been applied strongly as agents of drug delivery [14–17], vaccine delivery [18–20] and gene delivery [21, 22].

Bacterial resistance to antibiotics has been addressed by looking for new antibiotics and modified antibiotics. Unfortunately, the development of new antibiotics can not catch up to the microbial pathogen's fast and frequent development of resistance in a timely manner. Antimicrobial nanomaterials have been one of the recent ways to resolve this challenge, and microbial pathogens may not be able to develop the resistance and a novel nanosized form for efficient delivery. The antibacterial activity of chitosan nanoparticle–antibiotic complexes as nanoantibiotics has been recently reported. Chitosan nanoparticles prepared by an ionic gelation method with tripolyphosphate (TPP) exhibited antibacterial activity much more than a chitosan solution and doxycycline antibiotics [23]. The antibacterial activity of oleoyl–chitosan nanoparticles was also reported [24]. Anal et al. [25] also investigated the preparation of ionotropic cross-linked chitosan microspheres with TPP for controlled release of ampicillin, and the minimum inhibitory control (MIC) of the microspheres with *Staphylococcus aureus* were 12.5 μg/mL and prolonged for more 24 h. Preparation of chitosan microspheres was reported by Cevher et al. [26]. In this research, chitosan microspheres containing vancomycin were prepared by a spray drying method and it was found that the microspheres loaded with vacomycin were more effective than the intramuscular injection of vancomycin [26]. Jafarinejad et al. [27] developed chitosan nanoparticles for pulmonary delivery of itraconazole by using ionic

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gelation and a spray drying method. Ngan et al. [28] also reported preparation of chitosan nanoparticles by a spray drying method and loaded amoxicillin. The results showed that the antibacterial activity of chitosan nanoparticle–amoxicillin complex against *S. aureus* was two times more effective than the amoxicillin only [28].

The aims of this work are to determine the effect of chitosan molecular weight and nozzle size on average size, size distribution and zeta potential values of the chitosan nanoparticles, and to evaluate the antibacterial activity of the chitosan nanoparticles and chitosan nanoparticle–amoxicillin complex on *S. pneumonia.*

Experimental

Materials

Chitosan was purchased from Taehoon Bio, Seoul, Korea. The degree of chitosan deacetylation was approximately 80–90 %, as determined by infrared (IR) spectroscopy [29]. The molecular weight of chitosan was determined via its viscosity (Brookfield viscometer): very high molecular weight (VHMW) 1200 cPs (centipoises); high molecular weight (HMW) 760 cPs; medium molecular weight (MMW) 276 cPs; and low molecular weight (LMW) 130 cPs.

Streptococcus pneumoniae was obtained from the Institute of Hygiene and Epidemiology of Tay Nguyen University, Buon Ma Thuot, Dak Lak Province, Vietnam. TPP and acetic acid were obtained from Merck (Germany), and amoxicillin trihydrate was obtained from Purimox (India).

Preparation of chitosan nanoparticles

Chitosan nanoparticles were prepared according to a modified ionic gelation method described by Gan et al. [21]. In brief, chitosan with various molecular weights (130, 276, 760 and 1200 cPs) was dissolved in 0.35 % (w/v) acetic acid solution and kept over night at room temperature. The chitosan solution was adjusted to pH 5.5 by 0.5-M NaOH solutions. 0.25 % (w/v) TPP solution was dissolved in deionized water. And then, chitosan nanoparticles were prepared by dropping 0.25 % TPP solution into the chitosan solution at a chitosan:TPP ratio of 6:1 (w/w) while stirring at 900 rpm with a magnetic stirrer for 60 min at room temperature. The suspensions were centrifuged at 5000 rpm to remove precipitants and then the chitosan nanoparticles were collected by a nano spray dryer (Buchi Nano B90, Switzerland) placed in a clean bench room with three differently sized spray drying nozzles (4.0, 5.5 and 7.0 μm); the flow rate was 120 ml min^{-1}; the drying gas flow was 1.3 m^3 min^{-1}; the inlet temperature was 120 °C; and the outlet temperature was 80 °C. The chitosan nanoparticles were packed in glass tubes and stored at 5 °C.

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Particles size, zeta potential value and morphology of chitosan nanoparticles

The morphologies of the chitosan nanoparticles were investigated by field emission scanning electron microscopy (FE-SEM; model JSM 7401F, JEOL, Ltd., Japan) at an acceleration voltage of 15 kV and transmission electron spectroscopy (TEM; JEM-1400, JEOL, Ltd., Japan). Zeta potential value and size distribution of the chitosan nanoparticles were determined by a Zetasizer Nano ZS (Malvern, UK).

Evaluation of the antibacterial activity of chitosan nanoparticles

Streptoccocus pneumoniae was cultured in a brain heart infusion broth (BHI) with or without 1.5 % agar (Difco Laboratories, Detroit, MI, USA). *Streptoccocus pneumoniae* were grown in liquid BHI medium (pH = 7.0) supplemented with 60 μg/mL of chitosan nanoparticles with different molecular weight: 130, 276, 760 and 1200 cPs at 37 °C under 5 % CO_2 for 24 h. The growth inhibition of the bacteria by the chitosan nanoparticles was evaluated by a turbidity method at 610 nm. The experiment was conducted with five tests at 130, 276, 760, 1200 cPs and a control (the medium only) in triplicate, three tubes for each test.

Evaluation of antibacterial activity of chitosan nanoparticle–amoxicillin complex

The chitosan nanoparticle that performed the best in the above antibacterial activity test was used for this test. The liquid BHI medium (pH = 7.0) was supplemented with 60 μg/mL of chitosan nanoparticles loaded with amoxicillin at doses of 0, 5, 10, 15, 20 and 25 μg/mL. The bacteria were inoculated at 37 °C for 24 h under 5 % CO_2. The growth of the bacteria was determined by a turbidity method at 610 nm. The experiment was performed with three tests with chitosan nanoparticles only; amoxicillin only and the chitosan nanoparticle–amoxicillin complex were tested in triplicate, three tubes for each test.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Duncan's multiple range tests (triplicate) by MSTATC software. A P value ≤ 0.05 was considered statistically significant.

Results and discussion

The effects of the molecular weight of chitosan on the characteristics of chitosan nanoparticles prepared by TPP ionic gelation combined with a spray drying method and their resulting antibacterial activities are discussed herein.

Previous works reported that the size and zeta potential of chitosan nanoparticles and their antibacterial activities depended strongly on the molecular weight [9, 21, 28]. Therefore, in

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this work, four different molecular weights of chitosan were used for preparation of chitosan nanoparticles and evaluation of their antibacterial activity on *S. pneumonia.*

The results, listed in Table 1, show that the size and zeta potential value of chitosan nanoparticles prepared by TPP ionic gelation method combined with spray drying depended clearly on the molecular weight. Increasing the molecular weight of chitosan material from 130 to 1200 cPs led to an increase in the average size of the nanoparticles from 166.7 to 1230 nm and a decrease in the zeta potential value from 54.7 to 34.9 mV. The results were better than those in previous works [21, 28, 30]. Gan et al. [21] prepared chitosan nanoparticles by TPP ionic gelation from low-, medium- and high-molecular weight chitosan. The results showed that when increasing the molecular weight of chitosan, the size of the particles increased from 136 to 1628 nm and zeta potential decreased from 48.3 to 14.3 mV [21].

The size distribution and zeta potential value of the chitosan nanoparticles are shown in Figs. 1 and 2. The results shown in Fig. 1 indicated that all peaks of size distribution in various molecular weights were sharp. It means that the size distribution of the nanoparticles was narrow and the particles were the same size and uniform. These results are also demonstrated by TEM and SEM in Figs. 5 and 6.

The results shown in Table 1 and Fig. 2 also indicated that the chitosan nanoparticles prepared by the method possesed values of zeta potentials 34.9 to 54.7 mV and higher than the chitosan nanoparticles prepared by spray drying in our previous work [28]. The results shown in Table 1 and Fig. 1 also indicated that higher molecular weight of chitosan was higher size and lower zeta potential of chitosan nanoparticles. The mean size of the chitosan nanoparticles were 166.7 nm, 334.6 nm and 1230.0 nm at molecular weight of chitosan of 130, 760 and 1200 cPs. In general, the results indicated that the size distribution, zeta potential value and morphology of the chitosan nanoparticles prepared by TPP ionic gelation combined with a spray drying method in this work were better than the previous works [26–28, 30–34]. Dudhani and Kosaraju [32] prepared chitosan nanoparticles from low-molecular

Table 1 Relationship between the molecular weight of chitosan and the mean size, zeta potential of the nanoparticles and their antibacterial activity

Viscosity molecular weight (cps)	Mean size of the nanoparticles (nm)	Zeta potential of the nanoparticles (mV)	OD 610 nm
130	166.7	54.7	0.078 ± 0.0069^{a}
276	189.7	47.7	0.089 ± 0.0018^{b}
760	334.6	38.6	0.115 ± 0.0060^{c}
1200	1230.0	34.9	0.127 ± 0.0146^{d}
Control[f]	–	–	0.336 ± 0.0074^{e}

The bacteria medium was supplemented with 60 μg/mL of chitosan nanoparticles with viscosity molecular weights of 130, 276, 760 and 1200 cPs. The bacteria were inoculated at 37 °C for 24 h under 5 % CO_2. The growth of the bacteria was determined by a turbidity method at 610 nm. Data are means of three replicates. Superscripts a, b, c, d and e are ranges when comparison with an LSD 0.05 value (least significant difference at alpha 0.05). Different superscripts in the same column are significantly different between the treatments at a 5 % level according to Duncan's multiple range test

OD optical density

[f] Control: no addition of chitosan nanoparticles into medium of the bacteria

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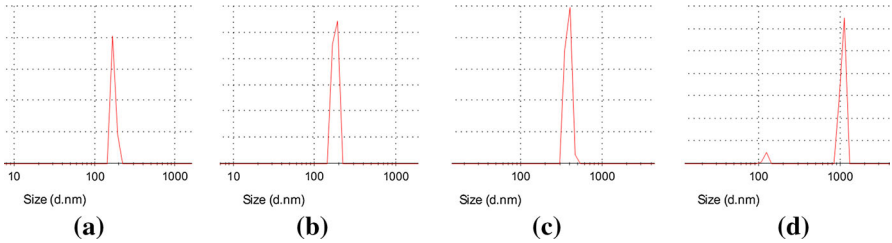

Fig. 1 Effect of various molecular weights of chitosan on the size distribution of chitosan nanoparticles prepared by TPP ionic gelation combined with a spray drying method under conditions: molecular weight of chitosan: 130 cPs (**a**); 276 cPs (**b**); 760 cPs (**c**) and 1200 cPs (**d**) at nozzle size 5.5 μm. Chitosan solution 0.1 % (w/v) in acetic acid 0.35 %, pH of the chitosan solution: 5.5 adjusted by NaOH 0.5 M; TPP (Tripolyphosphate) solution 0.25 % (w/v) in deionized water. The spray dryer was operated at a flow rate of 120 mL min^{-1}; the drying gas flow rate was 1.3 m^3 min^{-1}; the inlet temperature was 120 °C; and the outlet temperature was 80 °C

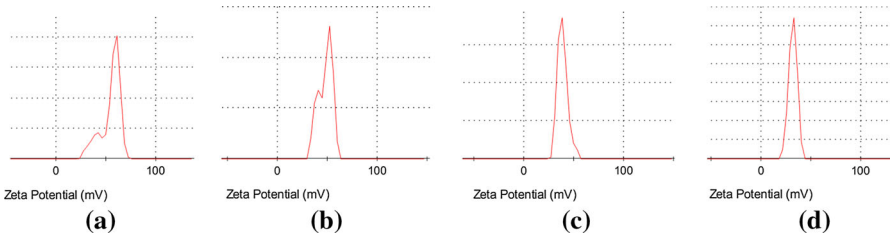

Fig. 2 Effect of various viscosity molecular weights of chitosan on zeta potential of chitosan nanoparticles prepared by TPP ionic gelation combined with a spray drying method. Molecular weight of chitosan: 130 cPs (**a**); 276 cPs (**b**); 760 cPs (**c**); 1200 cPs (**d**); and at a nozzle size of 5.5 μm

weight chitosan (20–200 cPs) by a TPP ionic gelation method; the weight ratio of chitosan to TPP was 2:1. Dudhani and Kosaraju reported that size distribution of the nanoparticles were very high, ranged from 20 to 600 nm and the zeta potential was only 31 mV [32]. Kašpar et al. [34] used the same method to prepared chitosan nanoparticles with low-molecular weight chitosan. The size distribution of the chitosan particles was so big, from 1 to 8 μm, and the particles were nonuniform and not smooth. But the zeta potential of the particles ranged only from 20 to 24 mV [34].

The antibacterial activity of the chitosan nanoparticles shown in Table 1 indicated that chitosan nanoparticles can inhibit strongly the growth of *S. pneumoniae* at a concentration of 60 μg/mL. The turbidity (OD 610 nm) of the test supplemented 60 ug/mL of chitosan nanoparticles with 130 cPs molecular weight was 0.078 compared to 0.336 in the control. The obtained results showed that higher molecular weight of chitosan nanoparticles and lower antibacterial activity on *S. pneumoniae*. Previous works reported that the antibacterial activity of chitosan depends on the polycationic charge on the chitosan molecule, higher positive charge and higher antibacterial activity [8, 9, 11]. The results shown in Table 1 demonstrated that chitosan nanoparticles prepared from low-molecular weight chitosan (130 cPs) had the highest zeta potential value (54.7 mV) and introduced highest antibacterial activity. These results were similar to some previous works [28, 35]. Fernandes et al. [35]

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studied the effect of the molecular weight of chitosan (625, 590 and 107 kDa) and a chitosan oligomer (1, 3 and 5 kDa) on antibacterial activity on *E. coli* and *S. aureus;* Fernandes reported that lower molecular weight chitosan and higher antibacterial activity [35]. Ngan et al. [28] prepared chitosan nanoparticles by spray drying with molecular weights of 276, 760 and 1200 cPs. The average size of particles was from 95 to 335 nm; a decrease in zeta potential values from 45.7 mV to 29.6 mV was observed, and an increase in the turbidity of the bacteria's culture (*S. aureus*) from 0.014 to 0.065 at 20 μg/mL was also reported.

Effect of nozzle size on characteristics of the chitosan nanoparticles and their antibacterial activity on *Streptococcus pneumoniae*

In this experiment, chitosan nanoparticles were prepared by ionic gelation combined with a spray drying method with 130-cPs chitosan material; other conditions were the same as the above experiment, but with different nozzle sizes: 4.0, 5.5 and 7.0 μm. The results shown in Table 2 indicate that using different nozzle sizes affects the size of the chitosan nanoparticles and their zeta potential values. Although the nanoparticles were formed by TPP ionic gelation, the size of the particles may be changed in the spray drying step (Figs. 3–6). Increasing the nozzle size from 4.0 to 7.0 μm resulted in an increase in the average size of the particles from 156.1 to 376.1 nm, and a decrease in zeta potential values from 59.3 mV to 50.0 mV. However, these changes were not as high as the above experiment. These results were similar to those of Kašpa's work [34], in which chitosan particles were prepared by the same method with two steps: TPP ionic gelation and spray drying with 1.4- and 1.5-mm nozzle caps. The results showed the size of nozzle cap clearly affected the size of the particles and zeta potential values. The size distribution of the particles ranged from 0.7 to 7.0 μm with a 1.4 mm nozzle cap, and increased to 3.0–9.0 μm with a 1.5-mm nozzle cap, much bigger than our results. Zeta potential values were 20 to 24 mV, lower than those of this article [34].

The antibacterial activity of the chitosan nanoparticles (Table 2) showed that the activity depends on the zeta potential value of chitosan nanoparticles. The particles had higher zeta potential values and higher growth inhibition of the bacteria (Figs. 5, 6).

Table 2 Effect of nozzle size on characteristics of the nanoparticles prepared by ionic gelation combined with a spray drying method

Observed data	Nozzle size (μm)		
	4.0	5.5	7.0
Mean size (nm)	156.1	186.7	376.1
Zeta potential (+mV)	59.3	54.7	50.0
Antibacterial activity (OD 610 nm)	0.053 ± 0.0009	0.0625 ± 0.0010	0.0792 ± 0.0025

Chitosan nanoparticles prepared by ionic gelation combined with a spray drying method under conditions: 0.1 % chitosan solution with 130 cPs; 0.25 % TPP; the ratio of chitosan:TPP was 6:1 (w/w); chitosan nanoparticles were collected by a nano spray dryer at a flow rate of 120 mL min^{-1}; the drying gas flow rate was 1.3 m^3 min^{-1}; the inlet temperature was 120 °C and the outlet temperature was 80 °C at nozzle sizes of 4.0, 5.5 and 7.0 μm

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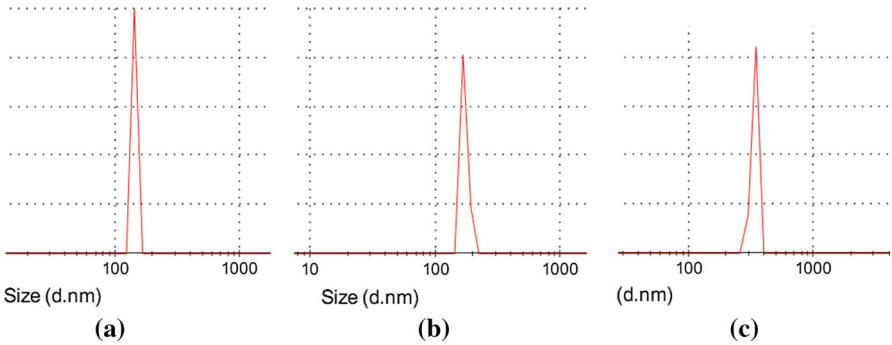

Fig. 3 Effect of nozzle size on the size distribution of chitosan nanoparticles prepared by TPP ionic gelation combined with a spray drying method. Molecular weight of chitosan: 130 cPs. Chitosan solution 0.1 % (w/v) in acetic acid 0.35 %, pH of the chitosan solution: 5.5 adjusted by NaOH 0.5 M; TPP (Tripolyphosphate) solution 0.25 % (w/v) in deionized water. The spray dryer was operated at a flow rate of 120 mL min^{-1} ; the drying gas flow rate was 1.3 m^3 min^{-1}; the inlet temperature was 120 °C; and the outlet temperature was 80 °C with various nozzle sizes: 4.0 μm (**a**); 5.5 μm (**b**) and 7.0 μm (**c**)

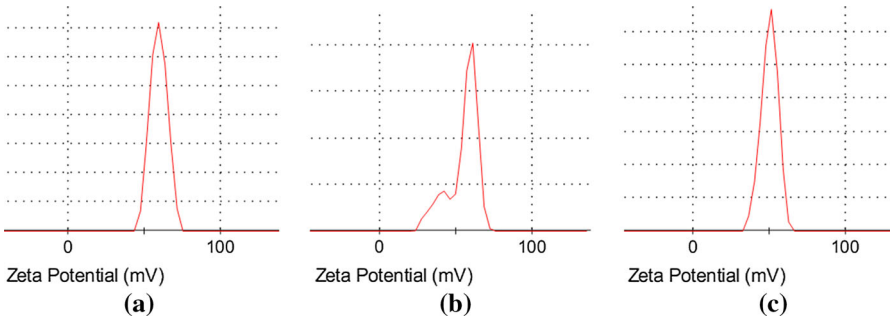

Fig. 4 Effect of nozzle size on zeta potential of chitosan nanoparticles prepared by TPP ionic gelation combined with a spray drying method under conditions: 0.1 % (w/v) chitosan solution 130 cPs was dissolved in acetic acid 0.35 %, pH of the chitosan solution was 5.5 adjusted by NaOH 0.5 M; TPP (tripolyphosphate) solution 0.25 % (w/v) in deionized water. The spray dryer was operated at a flow rate of 120 mL min^{-1}; the drying gas flow rate was 1.3 m^3 min^{-1} ; the inlet temperature was 120 °C; and the outlet temperature was 80 °C with various nozzle sizes: 4.0 μm (**a**); 5.5 μm (**b**) and 7.0 μm (**c**)

Antibacterial activity of chitosan nanoparticle-amoxicillin complex

60-μg chitosan nanoparticles with 130 cPs molecular weight were mixed with 5, 10, 15, 20 and 25 μg/mL amoxicillin to make the complex. The results showed that chitosan nanoparticles with a molecular weight of 130 cPs were able to load over 90 % of the amoxicillin. The antibacterial activity of the chitosan nanoparticle–amoxicillin complex is shown in Fig. 7. The results indicate that amoxicillin can completely inhibit growth of *S. pneumoniae* at 25 μg/mL. The antibacterial activity of the chitosan nanoparticle–amoxicillin complex increased strongly in comparison to amoxicillin and chitosan nanoparticles alone. The bacteria could be inhibited completely at 7.5 μg/mL of amoxicillin mixed with 60-μg chitosan nanoparticles. It

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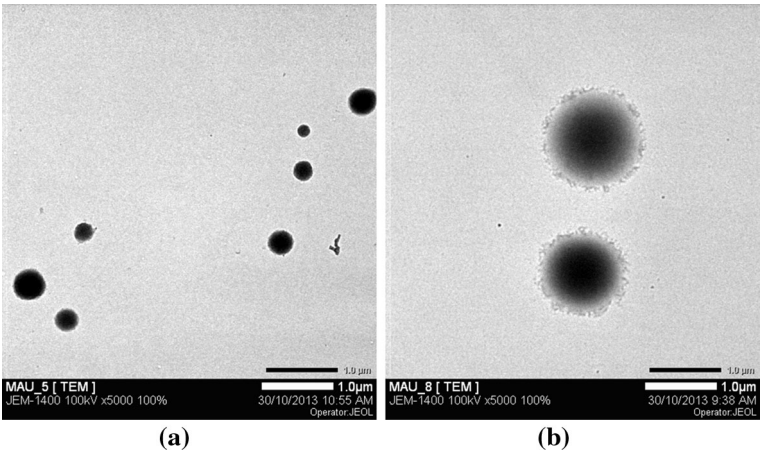

Fig. 5 TEM of of chitosan nanoparticles prepared by prepared by TPP ionic gelation combined with a spray drying method under conditions: 0.1 % chitosan solution 760 cPs (*left*) and 1200 cPs (*right*); 0.25 % TPP; the ratio of chitosan:TPP was 6:1 (w/w); chitosan nanoparticles were collected by a nano spray dryer (Buchi Nano B90, Switzerland) operated at a flow rate of 120 mL min^{-1}; the drying gas flow rate was 1.3 m^3 min^{-1}; the inlet temperature was 120 °C; the and outlet temperature was 80 °C with a nozzle size of 5.5 µm

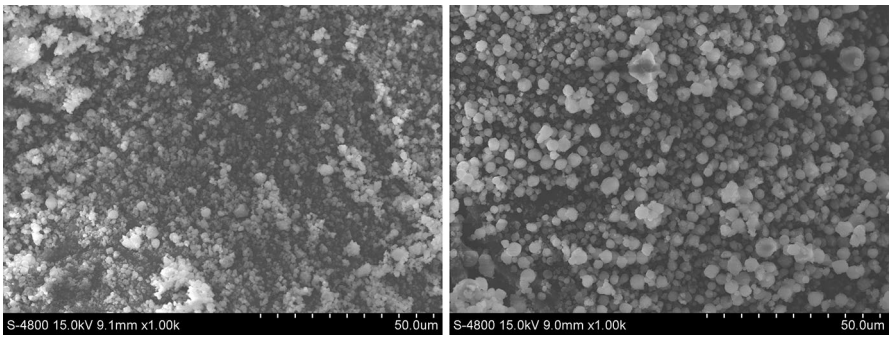

Fig. 6 SEM of chitosan nanoparticles prepared by prepared by TPP ionic gelation combined with a spray drying method under conditions: 0.1 % chitosan solution 130 cPs (*left*) and 1200 cPs (*right*); 0.25 % TPP; the ratio of chitosan:TPP was 6:1; chitosan nanoparticles were collected by a nano spray dryer operated at a flow rate of 120 mL min^{-1}; the drying gas flow rate was 1.3 m^3 min^{-1}; the inlet temperature was 120 °C; and the outlet temperature was 80 °C with a nozzle size of 7.0 µm

means the MIC value of the chitosan nanoparticle–amoxicillin complex on *S. pneumonia* is three times lower than the amoxicillin alone. The interactive effect of chitosan nanoparticles and antibiotics is similar to those reported by Anal et al. [25], Cevher et al. [26] and Ngan et al. [28]. The obtained results suggest that a chitosan nanoparticle–amoxicillin complex is a promising nanoantibiotic with advantages such as reduced side effects, low antimicrobial resistance, controlled drug release, low immunosuppression, improved solubility and low cost [36].

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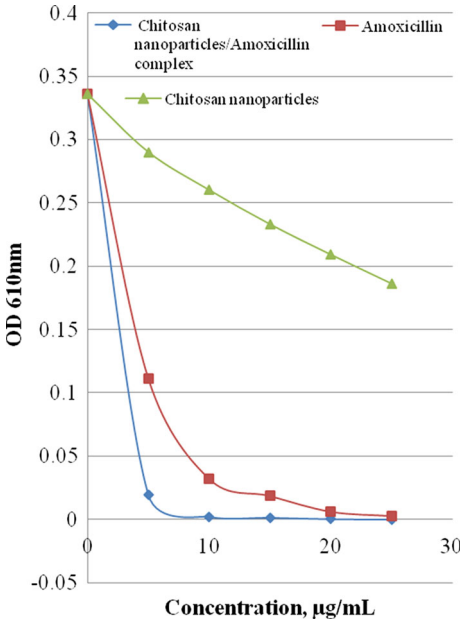

Fig. 7 Effect of a chitosan nanoparticle–amoxicillin complex on growth inhibition of *Streptococcus pneumoniae*. 130-cPs chitosan nanoparticles were loaded with amoxicillin dosages of 0, 5, 10, 15, 20 and 25 µg/mL and added into the cultivation medium of the bacteria. The bacteria were inoculated at 37 °C for 24 h under 5 % CO_2. The growth of the bacteria was determined by a turbidity method at 610 nm

Conclusions

The size and zeta potential value of chitosan nanoparticles prepared by TPP ionic gelation combined with a spray drying method are easily controlled by manipulating the molecular weight of the chitosan material and nozzle size. The size of chitosan nanoparticles ranges from 160 to 1230 nm with a homogeneous shape, and the zeta potential values range from 35 to 59 mV. The antibacterial activity of a chitosan nanoparticle–amoxicillin complex is three times stronger than the amoxicillin alone. It is concluded that a chitosan nanoparticle–amoxicillin complex is a promising nanoantibiotic.

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