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Chitosan microparticles loaded with *exotoxin A* subunit antigen for intranasal vaccination against *Pseudomonas aeruginosa*: An in vitro study

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ABSTRACT

Chitosan microparticles (CMs) were prepared with tripolyphosphate by spray-drying. Effects of polymer molecular weight, sonication power, cross-linking time and concentration of TPP on release profiles of catalytic or third domain *pseudomonas exotoxin A* (PEIII) and morphology of CMs were evaluated. The mean particle sizes of CMs were in the range from 1.09–1.46 μm and antigen loading efficiencies were more than 59%. As the molecular weight of chitosan increased, microparticles had a more spherical shape and a smooth surface. An increase in sonication power and decrease in cross-linking time resulted microparticles morphology changes.

Approximately 60–80% of PEIII released from microparticles within the first few hours. The release of antigen is increased significantly by raising the sonication power more than 45 W. When the cross-linking time extended from 15 to 60 min, the release of PEIII significantly reduced. The release of PEIII from the microparticles increased when concentration of TPP was raised.

1. Introduction

Pseudomonas aeruginosa, a gram-negative bacterial pathogen found mostly in water reservoirs, causes severe nosocomial and community acquired infections at a variety of body sites including the urinary tract, surgical or burn wounds, the cornea and the lower respiratory tract (Driscoll, Brody, & Kollef, 2007; Hauser & Rello, 2003). Patient groups at risk for acquisition of *P. aeruginosa* infections include those with hereditary diseases such as cystic fibrosis, paraplegic and burn patients, ones hospitalized in intensive care units and those undergoing mechanical ventilation, and patients immunosuppressed by certain diseases like cancer and AIDS. Although antibiotic therapy has considerably improved in the management of infectious diseases in general, many *P. aeruginosa* infections cannot be fully treated or eradicated by the application of anti-pseudomonal drugs and can thus establish chronic infections. Thus, vaccine development against *P. aeruginosa* may indeed be useful. Designs of *P. aeruginosa* vaccine are based on lipopolysaccharide, polysaccharide and polysaccharide-conjugate vaccines. Some other important methods include Flagella vaccines, outer membrane protein vaccines, killed whole cell and live-attenuated vaccines. While many experimental vaccines and monoclonal antibodies have been tested in preclinical trials, only a few have reached clinical phases and none of them has obtained approval. The lack of efficient *P. aeruginosa* vaccines may be overcome by using controlled release drug or gene delivery systems (Doring & Pier, 2008).

In relation to the different types of infection caused by *P. aeruginosa*-localized on mucosal surfaces such as the airways or systemic infection in the blood stream, one of the potential routes of administration of the vaccines is inhalation that could enhance their effectiveness. Concerning different routes of drug administration, intranasal drug delivery has many benefits, such as a large epithelial surface area produced by several microvilli, a porous endothelial membrane, and the ability to induce mucosal as well as systemic immunity. However, there are still limitations of nasal immunization, including limited diffusion of macromolecules across the mucosal barrier, enzymatic degradation within nasal secretions, and rapid clearance from nasal cavity, resulting in rapid systemic drug absorption (Kang et al., 2007).

In terms of peptide and protein delivery, natural polymers such as collagen, alginate and chitosan show great potential in use of vaccine delivery systems for pulmonary and intranasal delivery. Chitosan is a linear polysaccharide that is composed of randomly distributed D-glycosamine and N-acetyl-glycosamine units linked in a $\beta(1 \rightarrow 4)$ manner. Chitosan is used in multiple biomedical and pharmaceutical applications because of its bioavailability, nontoxicity, biocompatibility, biodegradability, high charge density, etc. (Kang et al., 2007). It has been shown that bioavailability of drug and carrier is raised by opening the tight junctions of epithelial cell layers and also reducing the rate of mucociliary clearance

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(Baumann, 2008). In addition to absorption-enhancing properties, interactions with the immune system may include the activation of macrophages and complement (Kang et al., 2007). More recently, chitosan-based delivery systems have also shown potential for nasal delivery of siRNA and subsequent gene interference in the lung mucosa, FGF-1 and FGF-2 (Csaba, Koping-Hoggard, & Alonso, 2009).

Microparticles and nanoparticles of chitosan and their solutions associated with influenza (CPMP, 1997; Jabbal-Gill, Fisher, Rappuoli, Davis, & Illum, 1998) and tetanus vaccine have been tested for inducing immunological responses following intranasal administration (Amidi et al., 2007). However, the delivery of antigens by mucosal path frequently results in a poor immune response (Czerkinsky et al., 1999; Holmgren, Czerkinsky, Lycke, & Svennerholm, 1992). These results have been attributed to several factors such as the limited diffusion of macromolecules across the mucosal barrier (Pereswetoff-Morath, 1998), rapid mucociliary clearance of drug formulations (Amidon, Flynn, & Donovan, 1990), and the presence of enzymatic degradation (Schipper, Verhoef, & Merkus, 1991). To overcome these problems, different strategies have been used, such as administration of antigens with mucosal adjuvants and/or entrapment of antigens into biodegradable microspheres/microparticles and liposomes (Sarkar, 1992).

The surface morphology, physiochemical properties and release characteristics of chitosan microspheres are related to preparation parameters, such as concentration and molecular weight of chitosan, pH and concentration of cross-linker agent, and curing time. The release of drug was highly dependent on the formulation variables, with significant interactions among these variables (He, Davis, & Illum, 1999; Ko, Park, Park, Hwang, & Park, 2003).

To the best of our knowledge, there is no study about using *exotoxin A* subunit antigen in an intranasal vaccine delivery system and influences of sonication power on the CMs characteristics. In this study, catalytic domain *exotoxin A* gene was cloned into pET28a as a expression vector and induced by IPTG as a inducer. The produced recombinant *catalytic domain exotoxin A* protein was determined with 12% SDS-PAGE. Finally, recombinant catalytic domain *exotoxin* A (rPEIII) was purified by MagneHis Protein Purification system kit and confirmed with immunoblotting. Also chitosan microparticles were characterized in terms of mean particle size, sphericity, loading efficiency and zeta potential. The morphology of microparticles and in vitro release behavior of the rPEIII of *exotoxin A* from the chitosan microparticles were studied as a function of formulation parameters such as cross-linker concentration, chitosan molecular weight and operational conditions such as sonication power and cross-linking time. Cross-linked microparticles were produced by spray-drying.

2. Materials and methods

2.1. Materials

Two types of chitosan with different molecular weight (MW) were used in this study. The low molecular weight chitosan (viscosity 75 mPa s in 1% acetic acid at 20 °C with a deacetylation grade of about 80%) and medium MW chitosan (viscosity 200 mPa s in 1% acetic acid at 20 °C with a deacetylation grade of about 80%) were respectively obtained from Chitotech Co. (Tehran, Iran) and Sigma–Aldrich Chemie (Steinheim, Germany). Tripolyphosphate (TPP) was obtained from Merck (Germany). Buffer substances and all other chemicals or solvents used were of analytical grade and purchased from Merck (Germany).

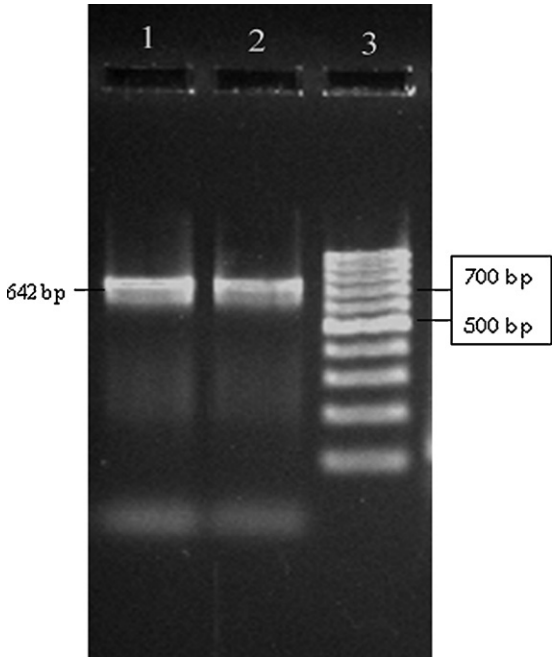

Fig. 1. PCR product of catalytic domain *exotoxin A P. aeruginosa* using genomic DNA, followed by agarose gel electrophoresis.

2.2. Antigen preparation

2.2.1. PCR amplification and cloning

First two primer pairs were designed for catalytic domain *exotoxin A* gene. PCR reaction performed using *Taq* DNA polymerase and *Pfu* DNA polymerase. The PCR product was digested by *EcoRI* and *HindIII* and purified by High Pure PCR Cleanup micro kit (Roche Applied Science). Furthermore, *pET28a* cloning/expression vector (Novagen) was digested with *EcoRI* and *HindIII*. The purified PCR product was inserted in the digested vector. The recombinant *pET28a-PEIII* plasmid was transformed into *E. coli* BL21 DE3. The host was grown overnight on the LB agar plate containing 80 mg/ml kanamycin.

2.2.2. Expression and purification

Five milliliters of LB Broth medium containing 50 mg/ml kanamycin was inoculated with 100 μl of the freezer stock of BL21 (DE3) cells with *pET28-PEIII*. After overnight growth at 37 °C, this culture was used to inoculate 100 ml of LB kanamycin medium in a 500 ml flask and the cells were grown at 37 °C with shaking until the A_{600} reached 0.6. At this point, 1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) was added to induce catalytic gene expression. The cells were then incubated at 37 °C for an additional 3 h. The induced cells were harvested by centrifugation at 5000 rpm at 4 °C for 15 min. The pellet was resuspended in 4 ml lysis buffer (50 mM Na–phosphate, pH 8.0, 300 mM NaCl, 5 mM benzamidine, and 0.5 mM PMSF) supplemented by protease inhibitor and lysozyme. The bacterial suspension was incubated at room temperature for 30 min to lyse cells. For purification, according to the manufacturer instruction, we used MagneHis protein purification system kit (Promega). Finally, recombinant protein was detected by Western blotting using Anti-His Taq kit (Roche Apllied Science).

2.2.3. Characterization

After *exotoxin A* gene amplification, a 642 bp DNA fragment produced (Fig. 1). Both PCR product and expression vector (*pET28a*) were digested by *HindIII* and *EcoRI* endonuclease, then ligated and transformed into *E. coli* Bl21DE3. The validity of cloning process

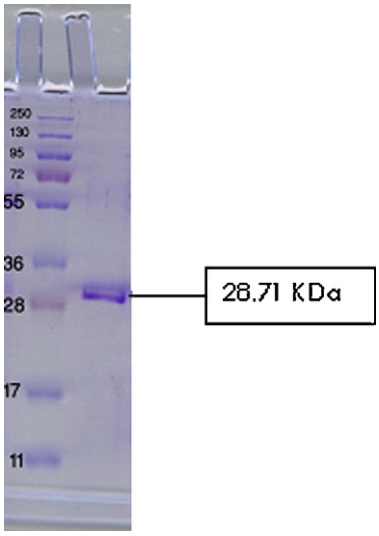

Fig. 2. Purification of the recombinant PEIII by MagneHis protein purification system kit. Protein was separated on a %15 SDS-PAGE gel and Commassie blue stained. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

was confirmed by digesting and sequencing of produced plasmids. Protein expression was induced with 1 mM IPTG at 37 °C for 3 h. The produced ~28.71 KDa recombinant catalytic domain *exotoxin A* protein was then analyzed and verified by 15% SDS-PAGE and western blotting analysis (Fig. 2) The assay revealed that the recombinant PEIII was strongly recognized by anti-His Taq, whereas no reactivity was observed in control (Fig. 3).

2.3. Preparation of chitosan–TPP microparticles

The chitosan–TPP microparticles containing an antigen were prepared by spray-drying method. Seven samples were prepared by spry drying method as follows: Chitosan was dissolved in 2% acetic acid solution (0.2 and 0.4 wt%). TPP was dissolved in water in order to make 1, 2 and 4 wt% solution while pH was adjusted to 4.0. Then, 20 ml of TPP solution was added drop wise to 200 ml of chitosan solution under the sonication power of 15, 45 and 90 W along with magnetic stirrers during 15, 30, 45 and 60 min. The suspension of cross-linked chitosan was then spray dried (Buchi® Mini spray drier, type 190, Switzerland) through a 0.2 mm nozzle at a feed rate of 2.4 ml/min. The driving pressure was 8 bar. Temperature was maintained at 120 °C. The microparticles were stored at 4 °C. Table 1 reports the code and composition of CMs.

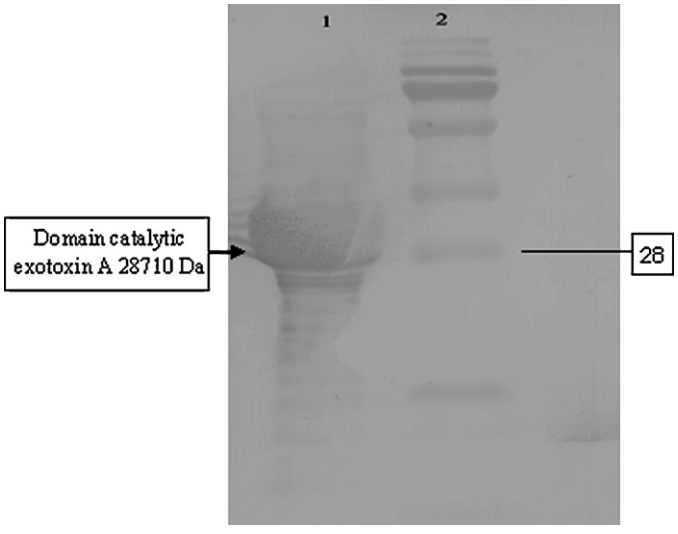

Fig. 3. Western blot of the PEIII. Line 1, PEIII protein with 6-His Taq. Line 2, protein marker.

2.4. Antigen loading

80 μg of the pure antigen was added to 1.5 ml of PBS buffer (pH 7.4) containing 5 mg of the chitosan microparticles which was prepared from different formulations. Then, suspension was kept at room temperature for 24 h under shaking to load the antigen by adsorption. After the loading, the suspension was centrifuged at 4000 rpm for 10 min to remove free, unloaded antigen. Then the microparticles were freeze-dried (MODULYOD freeze-dryer, Thermo electron Corporation). The concentration of unloaded antigen in the supernatant was determined by Bradford method (da Silva et al., 2005). The loading efficiency (LE) of antigen and loading capacity (LC) were calculated according to the following equations:

$$\mathrm{LE} = \frac{\text{Total amount of antigen} - \text{Free antigen}}{\text{Total amount of antigen}}$$

$$\mathrm{LC} = \frac{\text{Total amount of antigen} - \text{Free antigen}}{\text{Amount of chitosan microparticles}}$$

3. Characterization

3.1. Measuring particle size and zeta potential

Because of quick swelling of chitosan–TPP microparticles, their sizes could not be determined using laser diffraction in a particle size analyzer. Therefore, the particle size was determined utilizing scanning electron microscopy (SEM) images with specific software (Microstructure Measurement). For each sample, 300 particles are contributed in the measurement. Zeta-potential of the microparticles was determined by a Zetasizer Nano-ZS-90 (Malvern Instruments) in 0.1 mM KCl solution (pH 7.4) at 25 °C and automatic mode.

3.2. In vitro release studies

Release of PEIII from loaded CMs in vitro was carried out by the following method: five milligrams of antigen loaded microparticles were re-suspended in 1.5 ml PBS buffer (pH 7.4) and then placed in a thermostatic shaker (37 °C, 300 rpm). At predetermined intervals, the suspension was centrifuged (4000 rpm, 2 min) and 300 μl of supernatant was removed and replaced by the same quantity of PBS. The amount of the PEIII release was determined using the Braddford method (da Silva et al., 2005).

3.3. Morphology study

The shape and surface characteristics of the microparticles were observed by scanning electron microscopy (SEM). The microparticles were sputter-coated with thin gold layer and examined by means of a Philips SEM XL30 at 25 kV accelerating voltage.

3.4. Statistical analysis

Statistical analysis was performed using the computer based Excel program version 2007. All results were expressed as mean ± standard deviation (SD). Statistical differences between the groups were analyzed by Student's *t*-test. Differences were considered significant if probability values of $P < 0.05$ were obtained.

Table 1
Formulations of prepared samples.

Sample	Chitosan (wt%)	TPP (wt%)	TPP (pH)	Chitosan/TPP (v/v)	Cross-linking time (min)	Sonication power (*W*)	Polymer molecular weight
SI	02	1	4	10	60	45	Low
S2	0.2	1	4	10	60	45	Medium
S3	0.2	1	4	10	60	90	Medium
S4	0.2	1		10	60	15	Medium
S5	0.2	1	4	10	15	45	Medium
36	0.2	1	4	10	30	45	Medium
S7	0.2	1	4	10	45	45	Medium
S8	0.2	2	4	10	60	45	Medium
S9	0.2	4	4	10	60	45	Medium

4. Results and discussion

4.1. Microparticle size and zeta potential

Chitosan–TPP microparticles were prepared by the ionic interaction between a positively charged amino group of chitosan and a negatively charged of TPP molecules. TPP is a non-toxic and multivalent anion (Shu & Zhu, 2000). The ionization level of TPP is reliant at the pH value of solution. The TPP treatment of chitosan microparticles may improve their stability and their applicability in controlled drug delivery.

The properties of spray-dried chitosan–TPP microparticles (mean size, encapsulation efficiency, loading capacity and zeta potential) are illustrated in Table 2. It can be seen that the prepared CMs were extremely small and the mean particle sizes vary between 1.09 ± 0.34 and 1.46 ± 0.64 μm ($n = 3$). Formulation factors such as molecular weight, chitosan concentration, cross-linking time, TPP concentration and sonication power affected the size of CMs. The effects of these parameters were investigated by many researchers. For instance, the sonication power usually controls the particle size (Kabir, Saha, & Jeelani, 2007; Kang et al., 2006; Oosegi, Onishi, & Machida, 2008).

The above mentioned range of particle sizes seems appropriate for intranasal administration of vaccine. The main factors limiting pulmonary antigen delivery are poor deposition of the antigen at the alveolar area, low absorption from the epithelial barriers in the peripheral airways and the central lungs, and the presence of a mucociliary escalator in the central and upper lungs which rapidly removes antigens or particles from the central respiratory tract (Haynes, Shaik, Krarup, & Singh, 2004; Patton, Fishburn, & Weers, 2004). Mucoadhesive antigen-containing particles with an aerodynamic size between ca. 1 and 3 μm should be used to overcome these barriers. When particles are inhaled appropriately, they can penetrate deeply in the lung and have access to the alveoli and the mucosal associated lymphoid tissue (MALT) and are therefore attractive vaccine carriers (Haynes et al., 2004; Patton et al., 2004).

In this study, using a very low concentration of chitosan, employing ultrasonication technique as a high energy source and low and medium MW chitosan, produced fine microparticles.

Adsorption efficiency of antigen on CMs prepared from different formulation was presented in Table 2. Loading efficiency increased from 59.2% (S9) to 66% (S1). The highest Loading efficiency of *exotoxin A* (66%) was achieved by the formulation containing polymer with low molecular weight (S1) (Table 2). Statistical analysis shows there is no significant effect ($P > 0.005$) on changing the preparation factors in the samples based on medium molecular weight. Many factors may affect the loading efficiency of drug or biological agent in chitosan microspheres such as the nature of agent, concentrations of agent and chitosan, drug polymer ratio, amount of TTP solution and stirring speed as suggested (Kang et al., 2006).

Mainly, loading efficiency of protein by adsorption method is usually low. In adsorption method, protein is loaded on the surface of microparticles on the other hand its diffusion into the microparticles is limited. For example Wang et al. revealed that loading efficiency of insulin on chitosan microsphere was lower than 30%.

In the current study, the adsorption efficiency of PEIII in all formulations is between 59 and 67%, which implies suitable adsorption efficiency. Since chitosan was dissolved in aqueous acidic solution and it was strongly protonated, cationic molecules (chitosan) and anionic molecules (PEIII) were in the loading medium. Consequently, the anionic molecules strongly adsorbed onto the surface of cationic CMs. The high loading efficiency in CMs may be due to favorable interactions between the positively charged CMs and the negatively charged PEIII. Thus, most of the antigen is adsorbed near the surface of microspheres (Kang et al., 2006). The surface charge of CMs loaded with PEIII became lower than not loaded CMs which agree with the above assumption. The surface charge (zeta potential) of CMs and PEIII-loaded CMs was shown in Table 2.

The positive charges on the chitosan polymer can give rise to a strong electrostatic interaction with mucus or a negatively charged mucosal surface. This is to provide a longer contact time for drug transport across the nasal membrane, before the formulation is cleared by the mucociliary clearance mechanism (Illum, 2003; Singla & Chawla, 2001).

Table 2
Characteristics of CMs with PEIII.

Sample	Mean particle size (jim) ± SD	Zeta potential (mV) ± SD	Loading efficiency (yb) ± SD	Loading Capacity Oig PEIII/mg CMs)) ± SD
SI	1.09 ± 0.34	23.1 ± 1.2	66.0 ± 6	10J6 ± ±1.08
S2	1.30 ± 0.47	26.5 ± 1.4	62.2 ± 5.6	9.95 ± 0.96
S3	1.19 ± 0 26	24.3 ± 1.3	64.7 ± 4.1	1035 ± 0.66
S4	1.32 ± 0.7	25.6 ± 1.7	60.8 ± 3.9	953 ± 0.61
S5	1 25 ± 0.45	28.1 ± 1.5	66.3 ± 5.8	10 39 ± 0.91
M	1 23 ± 0 4	27.8 ± 1.2	61.4 ± 5.4	9 62 ± 0 85
S7	1 28 ± 0.53	26.9 ± 1.3	61.8 ± 4.6	9.68 ± 0.72
S8	1.41 ± 0.63	25 2 ± 1.8	62.1 ± 3.2	9.73 ± 0.50
S9	1.46 ± 0.64	25.1 ± 1.4	59.2 ± 3.7	9 25 ± 0.58
Chitosan (low MW)	–	28.7 ± 1.4	–	–
Chitosan (high MW)	–	33.8 ± 1.8	–	–

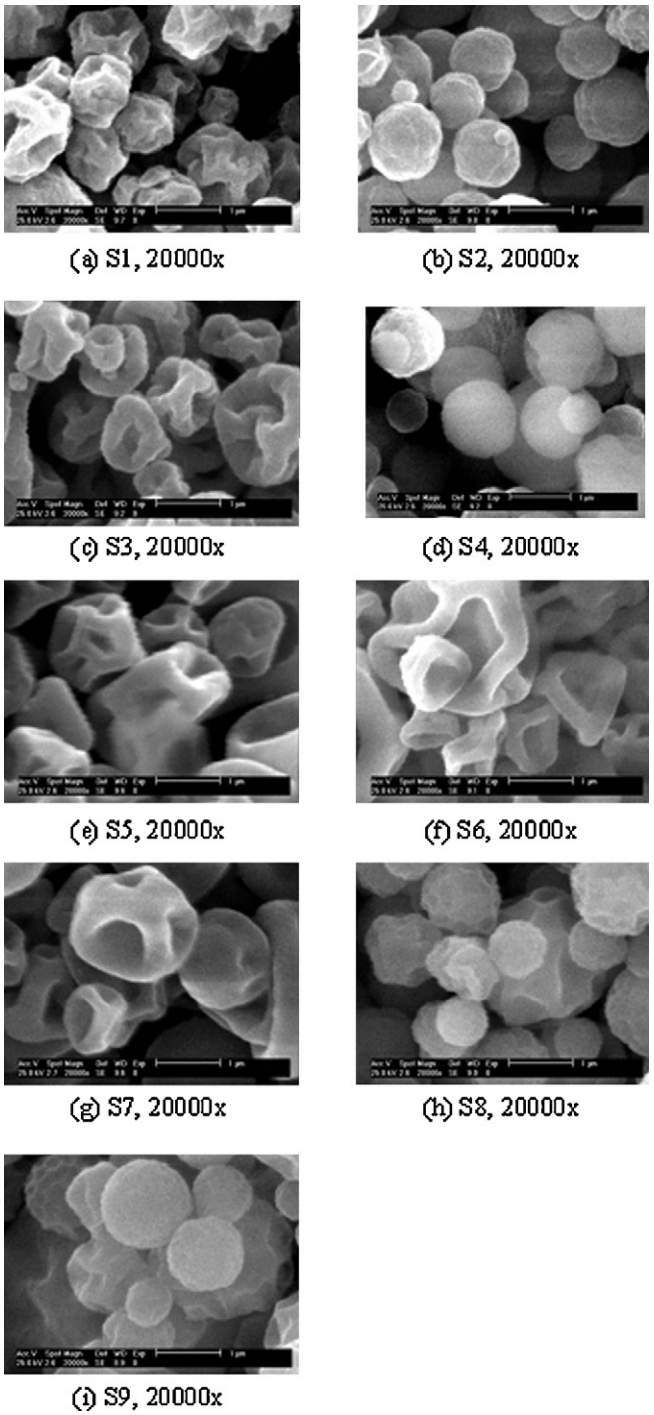

Fig. 4. SEM microphotographs of CMs prepared by spray drying method: (a) S1, (b) S2, (c) S3, (d) S4, (e) S5, (f) S6, (g) S7, (h) S8, (i) S9.

4.2. Morphology study

The surface morphology of the CMs is given in Fig. 4. It can be seen that most of the CMs have spherical or pseudo-spherical shape. Different parameters have great effects on the surface morphology of CMs.

4.2.1. Effect of molecular weight

It can be observed that the microparticles prepared from low molecular weight chitosan have pseudo-spherical shape and depressed surface, while those prepared from medium molecular weight have the more spherical shape and smooth surface (Fig 4a and b). The chitosan MW also affects the morphology of microparticles surface. When the MW of chitosan increased, the viscosity of chitosan solution increased and it may result in formation of relatively strong walls of microparticles upon interaction with TPP. Therefore, the higher the MW of chitosan, the more spherical the shape of microparticles (Ko, Park, Hwang, Park, & Lee, 2002).

4.2.2. Effect of sonication power

We found out from Fig. 4b–d that increasing of sonication power results in change in microparticles morphology from spherical shape and smooth surface in S4 to spherical shape and rough surface in S2 and irregular pseudo-spherical shape and wrinkled surface in S3. These results are in agreement with Dubey and Parikh. They also observed similar results in CMs by increasing the speed of mixing. It seems that sonication at high energetic processes declines microspheres wall stability (Dubey & Parikh, 2004; Ruan, Ng, & Feng, 2004; Yu, Wang, Hu, Li, & Tang, 2009).

4.2.3. Effect of cross-linking time

In the lower pH region, ionization degree of TPP and chitosan are high and chitosan forms gel with completely ionic-cross-linking without deprotonation (Lee, Mi, Shen, & Shyu, 2001; Shu & Zhu, 2001).

As the cross-linking time decreased, spherical shape of CMs transformed to irregular non-spherical shape (Fig. 4b, e, f and g). This result was similar to observations of Wang et al., who reported it when the cross-linking time of CMs with glutaraldehyde was less than 60 min, the reaction of functional groups of chitosan and glutaraldehyde was not enough to form a stable gel structure. Therefore, chitosan microparticle could not be formed (Wang et al., 2006b).

A high decline in cross-linking degree of chitosan due to a decrease in cross-linking time caused CMs to lose their ability to remain in spherical structure. It seems that one of the effective parameter on surface morphology is the stability of microparticle structure during the spray drying process. The solvent evaporation rate has a significant effect on microspheres surface morphology (Caliş, Bozdağ, Kaş, & Hincal, 1998).

As mentioned above, with decreasing of cross-linking time, the degree of chitosan cross-linking dramatically decreased which resulted in more compact chitosan microspheres and enhanced the solvent evaporation rate from droplets. Depressions observed on the surface may be due to the fast evaporation of water or high shear generated during the spray drying process. The results of Çalış and colleagues are in agreement with this aspect. They found that enhancement of the solvent evaporation rate causes the formation of porous and non-spherical BSA microspheres with wrinkled surface (Alpar, Somavarapu, Atuah, & Bramwell, 2005; Caliş, Bozdağ, Kaş, & Hincal, 1998; Xue, Yang, Zhang, & He, 2006).

4.2.4. Effect of concentration of cross-linking agent

Fig. 4b and h illustrates the surface smoothness and sphericity of microparticles decreased by the increase of TPP. Although it can be observed from Fig. 4h and i that there was no significant difference between surface morphology of samples S8 and S9. It may because the TPP could not diffuse to CMs and only stay on the outer layers. In a constant concentration of chitosan, with raising the cross-linking agent concentration, the most of residual cross-linking agent does not react with chitosan and therefore has no significant effect on the microspheres structure and characteristics (Wang, Gu, Su, & Ma, 2006a; Yuan et al., 2007).

The porous structure reduces the particle density, diminishing the aerodynamic diameter and increasing the propensity for deep lung deposition (Alpar et al., 2005; Vanbever et al., 1999).

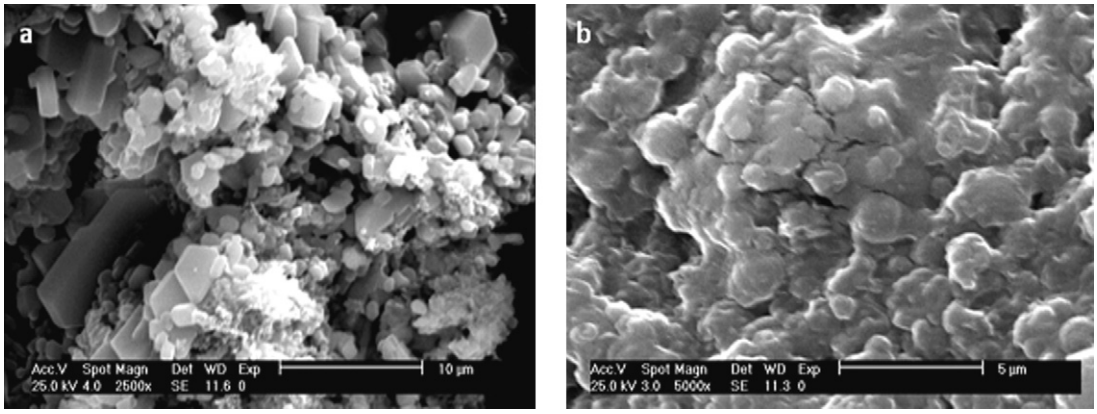

Fig. 5. SEM microphotographs of CMs prepared by spray drying method: (a) S6 and (b) S8 samples after 6 days incubation in PBS (pH 7.4).

The pores on the surface are supposed to be the result of rapid evaporation of solvent. During the solvent evaporation process, outer layer that is first formed on the surface of the droplets prevents the evaporation of the solvent which causes the rise of vapor pressure. Therefore, small explosion openings-pores are formed (Wang & Wang, 2002). Surface indentations could be attributed to the subsequent shrinking of the microparticles after solid outer layer is formed. This effect is especially apparent for samples S3, S5, S6 and S7 (Fig. 4c–g). Most of researchers reported that incorporation of drug in CMs when compared to free loaded microspheres had no influence on surface or morphological characteristics of microspheres prepared by different methods (Martinac, Filipovic-Grcic, Voinovich, Perissutti, & Franceschinis, 2005; van der Lubben et al., 2003).

4.3. Surface morphology after releasing PEIII

Fig. 5 shows typical scanning electron micrographs of surfaces of CMs after 6 days. Initially, CMs were observed to be spherical or pseudo-spherical. However, after six days, CMs were observed as aggregate shapes. This observation may be related to the neutral pH of release media (PBS pH 7.4) and long time of release study. It shows that the critical aggregation concentration (CAC) values of N-acyl chitosan (ALCS) decreased with the increasing of the pH value of the medium, indicating that the self-aggregation of acylated chitosans is affected significantly by the protonation extent of amino groups of the acylated chitosans (Jiang, Quan, Liao, & Wang, 2006). Therefore, in the neutral media most of chitosan molecules lose their electric charges and deposit from the media. On the other hand, the solution with higher pH than 6, amino groups of chitosan molecules give up their protons so van der Waals and hydrogen interactions increase as a result, a bulk of chitosan form and precipitate (Pepic, Filipovic-Grcic, & Jalsenjak, 2008).

4.4. In vitro release of PEIII from CMs

The release studies with different CMs were carried out in PBS at 37 °C with continuous shaking. In vitro release profiles of PEIII loaded CMs are given in Figs. 6–9. In vitro release studies indicated a biphasic antigen release pattern, characterized by a typical burst effect which followed by a slow release and continued for several hours. Approximately 60–80% of PEIII was released from microparticles within the first few hours.

4.4.1. Effect of molecular weight

Fig. 6 shows that higher molecular weight of chitosan released less antigen, but it is not significant ($P > 0.005$). It was probably because of increasing in smoothness and sphericity of CMs that caused the lower microparticles area with a lower release rate. It is reported that the higher the MW and degree of deacetylation of chitosan caused the lower release rate of salicylic acid from chitosan film (Puttipipatkhachorn, Nunthanid, Yamamoto, & Peck, 2001).

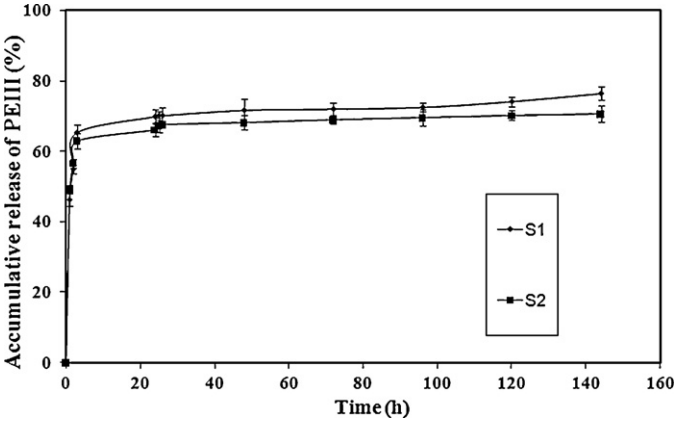

Fig. 6. Release profiles of PEIII-loaded CMs prepared with different molecular weight, low (S1) and medium (S2) molecular weight of chitosan. Mean ± S.D., n = 3.

4.4.2. Effect of sonication power

The antigen release studies of the samples S2, S3 and S4 are illustrated in Fig. 7. Based on these data, with increasing the sonication

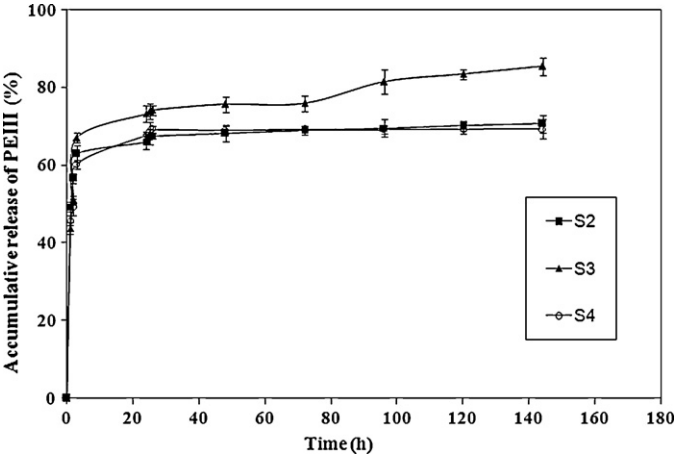

Fig. 7. The influence of sonication power on the PEIII release behavior from CMs. Mean ± S.D., $n = 3$.

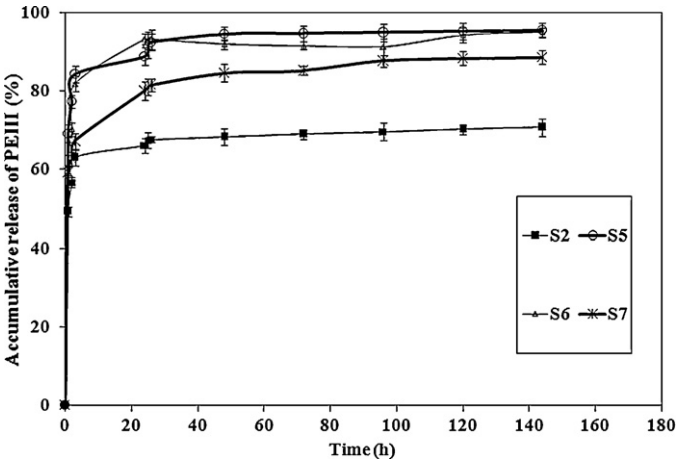

Fig. 8. The influence of cross-linking times on the PEIII release behavior from CMs. Mean ± S.D., $n = 3$.

power to more than 45 W, the release of the antigen significantly increased ($P < 0.005$). It may be related to a significant increase in the surface area of microparticles of sample S3. There is no significant difference between antigen release rate in samples S2 and S4 ($P > 0.005$) due to low alteration in microparticles surface morphology. It may be due to the fact that the area of chitosan microspheres has "drastic effect" on antigen release rate. These observations are similar with that reported by Xue et al. for TX46 release from chitosan microspheres (Ko et al., 2002; Xue et al., 2006). It seems that increasing of the microparticles surface caused more contact area with the release media and hence facilitated the release.

4.4.3. Effect of cross-linking time

Fig. 8 shows the release pattern of *exotixin A* loaded CMs prepared by spray drying. As cross-linking time increased from 15 to 60 min, the release of the antigen significantly decreased ($P < 0.005$).These results indicate that the CMs were cross-linked by TPP, depending upon its cross-linking time.

Previous studies have shown when chitosan solution was dropped into the TPP solution, the OH^- ions competed with tripolyphosphoric ions to react with amino group of chitosan immediately by ionic interaction and then $P_3O_{10}{}^{5-}$ ions diffused into chitosan drops to interact with amine groups of chitosan. Therefore, short curing time might not give the sufficient interaction time for the TPP-chitosan matrix (Ko et al., 2002; Mi et al., 1999).

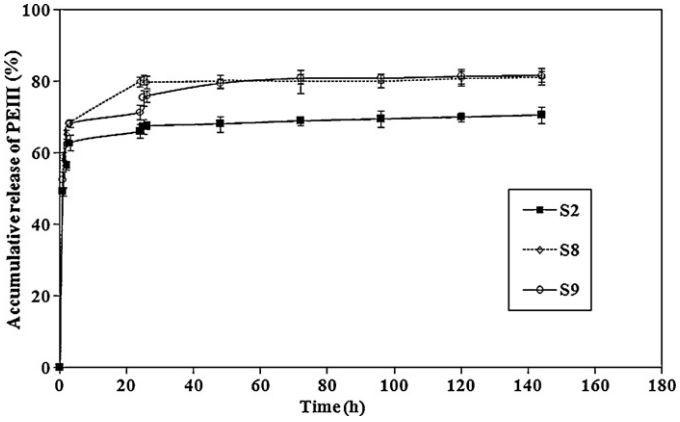

Fig. 9. The influence of concentration of cross-linking agent on the PEIII release behavior from CMs. Mean ± S.D., $n = 3$.

On the other hand, it seems that a considerable increase in the contact area of the microparticles with the release media has resulted in increase in the release rate due to the substantial increase in porosity, sphericity and surface smoothness of microparticles of the sample with the longer cross-linking time (Fig. 4). Since the porous structure results in an extensive surface area, large amounts of the antigen can be associated to the microparticles in this way.

4.4.4. Effect of concentration of cross-linking agent

Fig. 9 shows the effect of the cross-linking agent (TPP) amount on the antigen release behavior. In general, the release profile of drug from TPP-CMs decreased with the increasing of cross-linking agent concentration. Also it depends on the density of TPP–chitosan matrix. But in this study, as shown in Fig. 9, release of *exotixin A* was faster in the case of a higher concentrations of TPP (1–2 and 4 wt%) which was used for cross-linking. Statistical analysis shows there is significant difference ($P < 0.005$) between the formulations with low and high concentration of TPP. One reason for this behavior could be the increase of cross-linking degree which caused the CMs became more compact, so it was more difficult for antigen to penetrate into the inner layers of the microparticles resulting a larger fraction of adsorbed antigen localizes near the surface of CMs (Ko et al., 2002). These results were similar to the study of Xue et al. (2006). Similarly, the burst release of antigen from CMs, prepared with low concentration of TPP (1 wt%), was decreased to 66–67% within the first day when compared to the other samples (S8 and S9).

5. Conclusion

A variety of therapeutic agents such as anticancer, antiinflammatory, antibiotics, antithrombotic, steroids, proteins, amino acids, antidiabetic and diuretics have been incorporated in CMs to achieve controlled release. It is known that chitosan is characterised by absorption enhancing effects, because it improves the paracellular transport by opening the tight junctions. This biopolymer is one of the most important candidates for mucosal vaccine delivery. In this article, TPP–chitosan microparticles (CMs) prepared by spray drying is used as carriers for PEIII. The effect of various factors, especially effect of sonication power before spray drying of the chitosan solution, on the release behavior and morphology of the loaded microparticles were evaluated. The results show that the concentration of the cross-linking agent, time of cross-linking, MW and sonication power play important roles on the TPP–chitosan microparticles. As the MW was increased, the surface morphology of microparticles became more spherical and smooth. Variation of the sonication power, concentration of cross-linking agent and time of cross-linking resulted in changes in microparticles morphology from spherical shape and smooth surface to spherical shape and rough surface and irregular pseudo-spherical shape and wrinkled surface. However, this kind of microparticles needs additional improvements in the chemical structure such as tailoring chemical modification using functional groups to prevent of aggregation in release media. The release behaviors of the antigen are affected by sonication power, cross-linking time and concentration of TPP. Release of *exotixin A* was faster in the case of a higher concentration of TPP. This fact is very important that the normal mucociliary clearance time in healthy human's nose is about 20 min (Illum, 2003), whereas mucoadhesive chitosan microparticles were cleared from the human nasal cavity with a halftime of about 80 min. Therefore, this carrier system seems to be clinically acceptable for mucosal vaccine delivery to MALT.

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