Development of *Pasteurella multocida* loaded alginate based microspheres

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Abstract

Microencapsulation of Pasteurella multocida within alginate based microspheres (MSs) for subcutaneous vaccination was accomplished by emulsification-cross-linking technique. Alginate MSs were spontaneously formed by ionic cross-linking using calcium and zinc ions, and further stabilized employing zinc ion. A mean particle size of less than 10 um has been successfully developed using simple mixer and ultrasonic probe. The measured zeta potential of the MSs surface showed negatively charge of ~-30 mV indicating a strong negative charge at the particle's surface. A positively charge on MSs surface could be obtained by incubating with poly-L-lysine. Optical microscopy revealed spherical particles with uniformly distribution. The antigen entrapment efficacy of up to 75% was achieved. The slow release of antigen from MSs was observed up to a 6 month period.

Keywords: Pasteurella multocida, vaccine, Haemorrhagic Septicaemia, microspheres

Introduction

Haemorrhagic septicaemia (HS) caused by *Pasteurella multocida (P. multocida)* is a fatal systemic disease in cattle and buffalos. The disease is prevalent in South and South East Asia, serotype B:2, and in Tropical Africa, serotype E:2. An outbreak of HS, usually acute, widespread, and virulently fetal, would cause economical damage and threaten the cattle export industry. To date, vaccination is the most effective mean to prevent *P. multocida* outbreak. Alum precipitated vaccines (APVs), inactivated vaccine, is the most widely used as it is simple to manufacture. However, APVs provide short term protection, 6 month, and post-vaccination shock has been reported up to 10%. In the contrary, oil adjuvant vaccines provide longer protection for 1 year. However, these vaccines have disadvantages of high viscosity and post-vaccination high fever in cattle, which make it unpopular among the field users. Some attempts have been done to reduce formulation viscosity, but such vaccines suffer from high number of inactivated cells (10^{10}-10^{11}) (Shah, Shah, & deGraaf, 1997; Verma & Jaiswal, 1997). We, therefore, take initiative to develop a new vaccine delivery systems which hopefully provides at least the same or higher immune protection which last for at least 1 year and possess lower viscosity which leading to lower side effect during and after injection.

Microencapsulation is a unique way to delivery antigens and facilitates their uptake into lymphoid tissue. One of the most common materials used to encapsulate antigens is alginate. It is generally regarded as safe (GRAS) by the FDA and finding widespread application in pharmaceutical industry. Alginate is a biodegradable, biocompatible and water soluble linear polysaccharide extracted from brown seaweed. It is composed of alternating block of 1-4 linked α-L-guluronic acid and β-D-mannuronic acid residue. The parenteral administration of alginate microspheres (MSs) has been shown to provoke immune responses (Bowersock et al., 1996; Espevik et al., 1993; Kidane et al., 2001; Suckow, Jarvinen, HogenEsch, Park, & Bowersock, 2002).

In this study, the alginate MSs for subcutaneous injection were prepared by emulsion-cross-linking technique which is modified from Kidane et al. (Kidane et al., 2002). The mean particle size of less than 10 μm could be successfully prepared without the aid of high pressure homogenizer. The physicochemical properties of MSs were characterized including morphology, mean particle size, zeta

potential and entrapment efficiency. The pattern of drug release was also investigated as well as the stability of the alginate MSs.

Materials and methods

1. Materials

Sodium alginate, medium viscosity, was purchased from Fluka (UK). Methyl cellulose (MC) was kindly supported by Dow chemical (Methocel®, Michigan, USA). Pluronic® L61 was obtained from BASF (New Jersey, USA). Olive oil was purchase from TCFF (Bangkok, Thailand).
Hydroxypropylmethylcellulose (HPMC) was purchase form Srichand united dispensary (Bangkok, Thailand). Calcium acetate was purchased from POCH SA (Gliwice, Poland). Zinc acetate was purchased from Univar (New South Wales, Australia). Sodium citrate was purchased from MERCK (Damstadt, Germany). A formalin-inactivated whole culture of *Pasturella multocida*, serotype B:2,5 was obtained from the veterinary biologics division, Ministry of agriculture and cooperatives, Thailand. Micro BCA protein assay kit was purchased from Thermo Scientific (Rockford, USA). All other chemicals and solvents were of analytical grade.

2. Methods

2.1 Preparation of alginate MSs and antigen loaded analogues

Antigen containing alginate MSs were prepared by emulsion-cross-linking method. The formalin-killed cultures ($3x10^9$ cells/mL) were washed with de-ionized (DI) water before used. One hundred microliters of an antigen suspension was mixed with 2%w/v alginate solution to a final concentration of 10%v/v. The alginate mixture was slowly added to oil phase, composed of 0.5%v/v of Pluronic® L61 in olive oil, while continuously stirred at 1500 rpm using a Heidolph mixer (RZR2021, GmbH & Co., Germany) with a marine impeller for 10 minutes. The ratio of alginate and oil phase was 1:4. The internal droplets were further reduced using ultrasonic probe (VCX130, Sonic & Material Inc., CT, USA), amplitude 50%, pulse 5-2 second for 15 sec. Then, cross-linking solution (4.2 % w/v calcium acetate and 10% w/v zinc acetate) was added drop wise to the emulsion while stirred at 1500 rpm. The ratio of alginate and cross-linking agent was 1:3. Finally, the resulting antigen MSs were harvested by centrifugation at 1500 rpm for 5 minutes. Then, MSs were washed three times with DI water and once with 10% ethanol. The antigen MSs were further stabilized by incubating with stabilizing agent in the volume ratio of 1:1 for 20 minutes. Then, the stabilized MSs were washed 3 times with DI water and subsequently adjusted to 20 mL with DI water. Finally, the MSs were autoclaved and kept at 4°C before used.

Microspheres were prepared with different processing parameters to study the effect of a number of variables on their physicochemical properties. Process parameters were varied as follows: types of polymers; alginate, alginate and HPMC, and alginate and MC; types of stabilizing agents; 1% $BaCl_2$, 1% $MgCl_2$, 1-3% Zn acetate and 1% $CaCl_2$; and types of suspending medium; DI water and 0.01 M PBS pH 6.5, 7.0 and 7.4. The ranges of these variable values were selected based on preliminary experiments. Empty MSs were prepared using the same procedure. All samples were prepared in duplicate.

2.2 Studies of alginate microspheres coated with cationic polymer

Alginate MSs were coated with chitosan and poly-L-lysine (PLL) to modified surface charge of particles. The volume ratio of alginate MSs suspension and cationic solution was at 1:1. Briefly, the alginate MSs suspension was incubated with polycationic solution for 20 min under continuously stirring at 600 rpm. Then, the particles were washed 3 times with DI water by centrifugation method.

2.3 Physicochemical characterization

The morphology of both unloaded and antigen loaded particles was investigated using scanning electron microscopy (SEM) (1455VP, LEO Electron Microscopy Ltd., Cambridge, UK). The mean particle size of the MSs was determined by an optical microscope (BX 50, New York, USA). At least 300 particles were measured using Feret's diameter. Cumulative percentage, frequency, undersize and normalised Z-value were calculated. In order to calculated the geometric mean diameter D50, particle diameter value was transformed into logarithm value.

The zeta potential of the MSs was determined by phase analysis light scattering employing a ZetaSizer (Nano ZS90, Malvern, UK). The

measurement angle was 90° to the incident light. Data were collected for 10 cycles. The zeta potential was calculated from the electrophoretic mobility using the Smoluchowski approximation approach (Tscharnuter, Mcneil-Watson, & Fairhurst, 1998)

2.4 Determination of antigen entrapment efficiency

Microspheres were incubated with 4% w/v sodium citrate solution at a ratio of 1:1 for 3 hr under mild stirring at 400 rpm. After particles dissolved, sample was centrifuged at 12,000 rpm for 5 min. The resulting pellets was washed 3 times with DI water and finally re-suspended in 1 mL of DI water. Then the amount of antigen was determined using the bicinchoninic acid (micro BCA) protein assay in 96 well plates. The samples were incubated at 37 ± 0.5°C for 2 h. Finally, the amount of antigen was determined at 570 nm using microplate reader (Ceres UV900C, Bio-Tek Instrument, USA). The calibration curve was performed in the range of $1-10x10^7$ cell/mL. The percent antigen entrapment was then calculated according to the equation:

$$\text{Antigen entrapment (\%)} = \frac{\text{amount of antigen in particle}^* 100}{\text{initial amount of antigen}} \quad (1)$$

2.5 *In vitro* antigen release studies

The *In vitro* release studies were performed by keeping samples in the dark at 37 ± 0.5°C for 6 months. Five hundred microliters of bacterin loaded MSs were placed in 0.5 mL of 0.2 M HEPES buffer, pH 7.4. At selected time interval, the amount of bacterin remained in the MSs was determined. The samples were centrigufed at 3,000 rpm for 5 min to get rid of released bacterin. Then, MSs were dissolved by incubating with 4% w/v sodium citrate at a ratio of 1:1 for 3 hr. Then the amount of antigen was determined using micro BCA protein assay in 96 well plates as described before. The percentage of bacterin released was determined by indirect method according to the equation:

$$\text{Antigen release (\%)} = 100 - \left[\frac{\text{amount of antigen in particle } *100\%}{\text{initial amount of antigen}}\right]$$

Results and discussions

1. Preparation of bacterin loaded and unloaded microspheres

The particles size and surface properties of MSs are crucial factors determining the uptake by phagocytic cell. It is well-known that particles less than 10 um and hydrophobic are best taken up by macrophages. In this study, alginate MSs were prepared by emulsification-cross-linking technique which was modified from Kidane et al., but without the use of high pressure homogenizer. We found that the mean particles size could be successfully reduced to less than 10 μm by using simple mixer and ultrasonic probe. In this technique, both the emulsification and cross-linking steps are crucial for desired particle size. First, w/o emulsion was prepared with the aid of mixer by dispersing alginate solution into oil phase. Then, the internal droplets were further reduced using ultrasonic probe during cross-linking step. Alginate MSs were spontaneously formed by ionic cross-linking using calcium and zinc ions. In the preliminary studies, we found that ultrasonic probe could help reduce the mean particle size. The particle prepared without and with the aid of ultrasonic probe showed a mean particle size of ~ 20 and ~7 μm, respectively. The hydrophobicity of microspheres could be modified by blending HPMC and MC with alginate (Kidane et al., 2002). Both MC and HPMC are more hydrophobic than alginate.

In the preliminary studies, the erosion of alginate MSs was observed under light microscope after kept in DI water for 1 month. Thus, to further strengthen the alginate MSs, the resulting MSs were incubated with different divalent cations; 1% $BaCl_2$, 1% $MgCl_2$, 1% Zn acetate and 1% $CaCl_2$, for 30 min. After centrifuged at 5000 rpm for 5 min, the re-dispersion ability of pellets was determined by rotate the centrifuge tube up and down 6 times, then the amount of precipitate in the system was determined. The results showed that alginate MSs incubated with

1% Zn acetate could be easily re-dispersed, while those MSs incubated with other hardening agents showed some aggregation suggesting weaker particles formation, Table 1. Thus, the results indicated that zinc acetate was the best stabilizing agent for alginate MSs. We further examined the optimal amount of zinc acetate as a stabilizing agent using the same experiment as mentioned. The 3% zinc acetate is the optimal hardening solution that could enhance the particle stability. Zinc and calcium ion were bind at different sites of alginate molecule. Calcium cations are commonly known to bind preferentially at the glulуronic acid unit, while zinc cations are less selective and hence produce more extensive cross-linking of alginate (Aslani & Kennedy, 1996).

Table 1. Re-dispersion ability of alginate microspheres after incubation with different stabilizing agents

Stabilizing agent	**Re-dispersion ability (the amount of precipitation after re-dispersion)**
Untreated microspheres	Moderate (moderate precipitation)
1 % $BaCl_2$,	Moderate (moderate precipitation)
1 % $MgCl_2$,	Good (less precipitation)
1 % $CaCl_2$	Moderate (moderate precipitation)
1% Zn acetate	Excellent (no precipitation)
3 % Zn acetate	Excellent (no precipitation)

2. Physicochemical characterization

The alginate based MSs, both bacterin loaded and unloaded MSs were examined by optical microscope and SEM. Regardless of types of polymers used, all of MSs formulations revealed spherical shape and uniformly distributed as illustrated by optical micrographs in Figure 1. The dark round-like structure seen in the MSs was attributed to the presence of olive oil. SEM micrograph also showed spherical particles with smooth surface, Figure 2

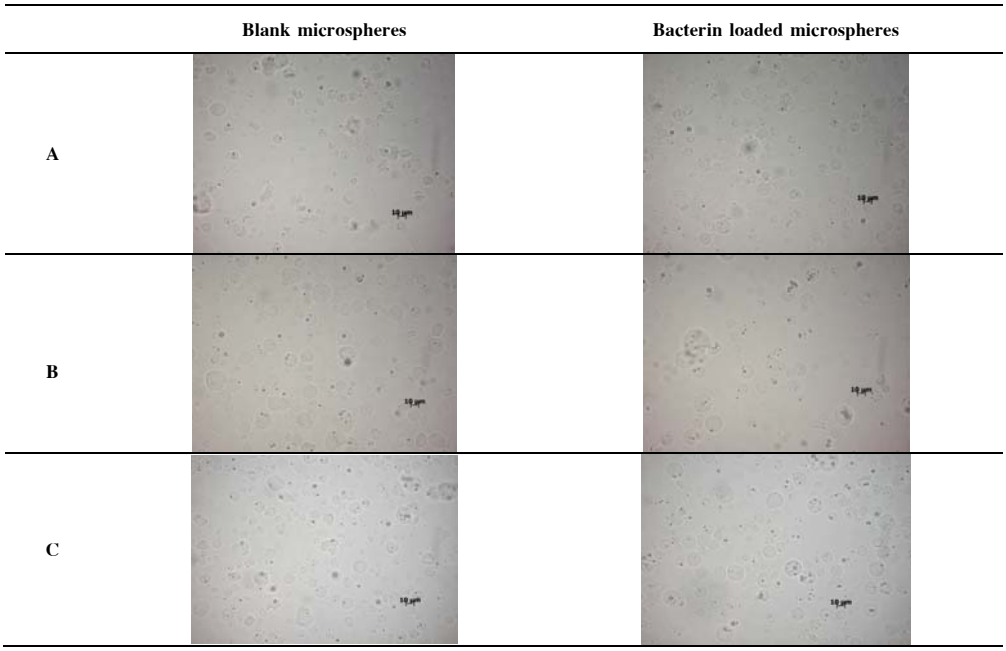

Figure 1. Optical photographs of bacterin loaded and unloaded microspheres, (400x magnification), prepared from (A) 2% alginate; (B) 2% alginate + 1% HPMC; (C) 2% alginate + 1% MC.

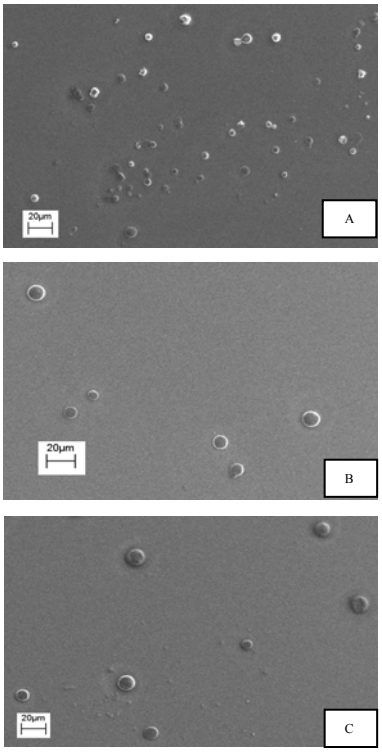

Figure 2. Scanning electron micrographs of bacterin loaded and unloaded microspheres prepared from (A) 2% alginate; (B) 2% alginate + 1% HPMC; (C) 2% alginate + 1% MC.

The mean particle size (D_{50}) and size distribution of both bacterin loaded and unloaded MSs were determined by optical microscope using Feret's diameter, Table 2. A unimodal size distribution in a range of 3-39 μm was observed. The MSs prepared form 2% alginate (A), 2% alginate and 1% HPMC (B), and 2% alginate and 1% MC (C) showed a mean particles size of ~ 7.6, 11.5, and 7.6 μm, respectively. The results suggested that alginate-HPMC MSs provided a bigger mean particles size than alginate and alginate-MC MSs. The alginate-HPMC and alginate-MC MSs showed broader particle size distribution than alginate MSs.

Table 2. Mean particles size and zeta potential of alginate based microspheres prepared with different polymers

Formulation	D50 (μm) ± SD		Zeta potential (mV) ± SD	
	Blank microspheres	Antigen loaded microspheres	Blank microspheres	Antigen loaded microspheres
A	7.3 ± 1.4	7.6 ± 1.3	-26.2 ± 0.8	-27.2 ± 1.0
B	9.4 ± 1.5	11.5 ± 1.3	-28.6 ± 2.3	-27.5 ± 1.1
C	7.6 ± 1.4	7.4 ± 1.5	-30.6 ± 1.6	-30.0 ± 1.2

Regardless of the types of polymers used, all formulations studied possessed the same zeta potential of ~ -30 mV suggested that alginate carboxylic group presented on the particle surface. Attempt to convert the surface charge of MSs form negative to positive had been done by incubating the alginate MSs with cationic polymer solutions, chitosan and poly-L-lysine (PLL), at various concentration. The zeta potential of MSs coated with 3% PLL possessed a positively charge of ~ +28 mV, while those MSs coated with lower concentration of PLL showed negatively charge of ~ -23 mV, Table 3. The higher amount of PLL could adsorbed cover the particles surface, therefore, converting the zeta potential from negative to positive charge. Nevertheless, the MSs coated with chitosan possessed a negatively charge, even when incubating with high amount of chitosan, 2% chitosan solution. Moreover, the MSs coated with chitosan solution tended to agglomerate as observed under light microscope.

Table 3. Zeta potential and appearance of alginate microspheres uncoated and coated with ply-L-lysine using optical an microscope with X400 magnification

	Appearance of microspheres after autoclave	Zeta potential (mV) ± SD
Uncoated alginate MPs	Whitish spherical particles with no aggregation	- 26.2 ± 0.8
Alginate MSs coated with		
- 0.5% Poly-L-lysine	Light brown color with fibrous structure and some precipitation	- 23.7 ± 5.4
- 1.0% Poly-L-lysine	Light brownish spherical particles with some aggregation	- 20.9 ± 4.9
- 3.0% Poly-L-lysine	Whitish spherical particles with no aggregation	+ 28.0 ± 1.6

3. Determination of antigen entrapment efficiency

The entrapment efficiency was determined by micro BCA assay. The results showed that formulation prepared with alginate revealed slightly higher entrapment efficiency than those formulated with alginate-HPMC and alginate-MC, respectively. The entrapment efficiency was ranged from 45-74%, Table 4. The moderate entrapment efficacy was attributed to the lost of antigen during washing step.

Table 4. Percentage of drug entrapment employing different types of polymer

Formulation	Entrapment efficiency (%) ± SD
A	74 ± 9
B	56 ± 11
C	45 ± 2

4. *In vitro* antigen release studies

The cumulative percent release of bacterin from alginate based MSs over varying time interval was examined. A sustained released profile over a period of 6 month was observed with all the formulation tested, Figure 3. In comparison, the blended alginate MSs showed higher amount of antigen released than alginate MSs. Within 6 month, ~ 40% and 18% of baterin were released from alginate blended MSs and alginate MSs, respectively. This result may be due to alginate MSs provide stonger particles than blended alginate MSs, as stabilizing agent was interacting with alginate. A sustained released characteristic suggested that kill bacterin were entrapped within the microspheres and may slowly released through the particle esosion. Thus, this prolong released characteristic of alginate based MSs system should provide a long term immune responses.

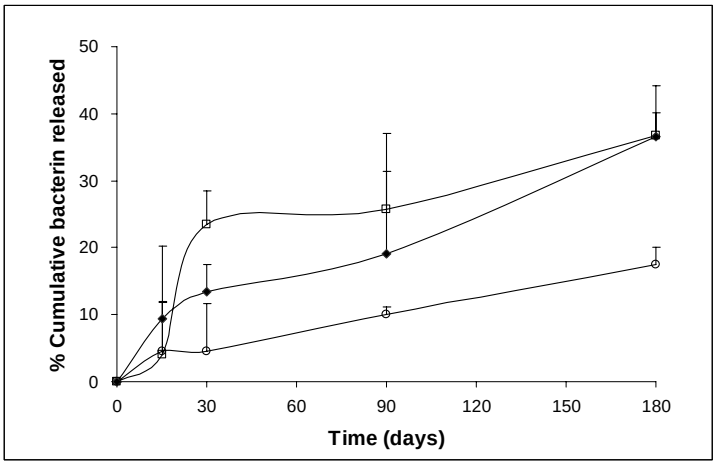

Figure 3. Release profiles of bacterin loaded and unloaded microspheres prepared from (O) 2% alginate; (♦) 2% alginate + 1% HPMC; and (□) 2% alginate + 1% MC.

Conclusions

Microencapsulation of *Pasteurella multocida* within alginate based microspheres for subcutaneous vaccination was successfully developed by emulsification-cross-linking technique. In this study, a preparation method using simple mixer and ultrasonic probe to produce MSs with a mean particles size of less than 10 μm has been developed without the aid of high pressure homogenizer. All formulation possessed a mean particles size of ~ 8 micron with narrow size distribution, ranging from 3-39 micron, which is suitable for vaccine delivery. The alginate based MSs showed negatively charge of ~ -30 mV suggesting high stability of particles. Nevertheless, the positively charge particles can be midified by incubating with poly-L-lysine. A moderate percentage of entrapment efficacy up to 70 was observed. Thus, It can be concluded that, given their numerous advantages and their ability to provide a sustained antigen release profile over a 6 month period, alginate based MSs can reasonably be considered as a promising platform for parenteral vaccine delivery system for *Pasteurella multocida*. Further animal experiments will be conducted to test the efficacy of this alginate MSs preparation.

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