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Original Article

EFFECT OF CROSS LINKING AGENT AND POLYMER ON THE CHARACTERISTICS OF OVALBUMIN LOADED ALGINATE MICROSPHERES

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ABSTRACT

Objective: The aim of the present research was to investigate effect of polymer and cross linking agent on the characteristics of ovalbumin-loaded alginate microspheres. Ovalbumin was selected as a protein model antigen; barium chloride and calcium chloride were used as cross linking agent.

Methods: Ionotropic gelation using aerosolisation and drop technique were applied in this study. The microspheres produce were characterized for the size, morphology, encapsulation efficiency, protein loading, yield and in vitro release. Release of the protein was also studied.

Results: Ovalbumin-loaded alginate microspheres were successfully produced by aerosolisation with maximum encapsulation efficiency and loadings of about 89%. Smooth and spherical microspheres were shown for both alginate microspheres produced using Ca²⁺ and Ba²⁺ of the aerosolisation method with average sizes from 12 to 30µm. In case drop technique, bigger microspheres size was produced of around 1-3 mm. The in vitro release study revealed that protein release decreased by decreasing alginate concentration, whereas no significant differences of ovalbumin release by decreasing calcium chloride concentration. Interestingly, alginate microspheres produced using barium chloride resulted burst and faster release behaviour of ovalbumin in HCl pH 1.2 and PBS pH 7.4 release medium.

Conclusion: This result suggested that modification of cross linking agent and polymer concentration were important for sustained release characteristics of ovalbumin-loaded alginate microspheres.

Keywords: Ovalbumin, alginate, Barium chloride, Calcium chloride, drop, Aerosolisation.

INTRODUCTION

It has been known that antigen stability was the main focus in oral delivery system. Oral vaccination is the most effective way to prevent most infectious diseases. Parenteral route has been an alternative route to administer vaccine due to vaccine instability when administered orally in GI tract and patient inconvenience. Thus prolonged immunity effect of vaccine delivery system has been developed [1].

Ovalbumin was a globular protein consist of 385 amino acids with molecular weight of around 45 kDa [2], was a model antigen which can enhance immune response. However, ovalbumin was a poor immunogenic need to administer several times to provide sustained release [3]. To overcome this obstacles, the need of experts and the easiest of uses were considered as well as to minimize side effect of vaccine [4]. Microencapsulation has been used to coat particle using polymer to produce microspheres of around 1 to 1000µm. Microspheres have potential in delivering and entrapping antigen to sustain release of antigen. Ionotropic gelation was selected as one of microencapsulation method based on polyelectrolyte ability to cross link counter ions forming hydrogel. Drop method and aerosolisation were used this technique. The advantages of both techniques were its ability to produce a simple, fast, non toxic and cost effective method [5]. In case of protein use, denaturation or stability issues of the protein could be avoided [5].

Alginate is a natural polysaccharide polymer consisting variations of guluronic and mannuronic acid units [6]. Sodium alginate is a non toxic material, biocompatible and biodegradable [7]. Gel was formed as a result of cross linking between polymer and divalency cations [8]. Ba²⁺ and Ca²⁺ were most commonly used ions to cross link with alginate polymer [9]. Effect of cross link agent and polymer was investigated in this research to characterize ovalbumin-loaded alginate microspheres. The microspheres were evaluated in terms of size, morphology, encapsulation efficiency, loading, yield and in vitro release.

MATERIALS AND METHODS

Materials

Ovalbumin *pharmaceutical grade* (Sigma-Aldrich Inc.); Sodium alginate *pharmaceutical grade* dan BaCl₂ *pharmaceutical grade* (Sigma-Aldrich Inc.); CaCl₂·2H₂O *pharmaceutical grade* (Solvay Chemicals International); Sodium citrate *pharmaceutical grade* (Weifang Ensign Industry Co. Ltd.); Na₂HPO₄ *pro analysis* (Merck); KH₂PO₄ *pro analysis* (Merck); NaCl *pro analysis* (Merck); HCl *pro analysis* (Merck); NaOH *pro analysis* (Merck); *Protein Quantification Kit* (Sigma-Aldrich Inc.); Aquadest.

Preparation of microspheres using aerosolisation technique

The alginate-ovalbumin solution was sprayed into cross linking agent solution and was stirred at 1000 rpm for 2 hours. Microspheres were washed by centrifugation at 2500 rpm for 6 minutes and washed twice using aquadest. Ovalbumin-loaded alginate microspheres were then collected and freeze dried at -80°C for 20 hours.

Preparation of microspheres using drop technique

The alginate-protein dispersion was added drop wise via burette in the crosslinker solution while stirring at 1000 rpm for 2 hours. Microspheres were washed by centrifugation at 2500 rpm for 6 minutes and washed twice using aquadest. Ovalbumin-loaded alginate microspheres were then collected and air-dried for 24 hours. Formulas of alginate microspheres were showed in table 1.

Characterization of alginate microspheres

Size and morphology: Size was determined by optical microscopy and morphology was investigated using Scanning Electron Microscopy (SEM).

Protein loading: Accurately added 50 mL of sodium citrate pH 8.5 in 400 grams of microspheres and was continuously stirred at

1000rpm for 12 hours. The absorbance of ovalbumin was measured using UV Vis Spectrophotometry at 600 nm using protein quantification kit sigma.

Encapsulation efficiency: The ratio of the actual ovalbumin content in the protein-loaded microspheres to the theoretical ovalbumin content was termed encapsulation efficiency.

Yield: Yield was calculated by percentage of total microspheres (grams) divided by total amounts of polymer and ovalbumin (grams).

In vitro release study

Release of ovalbumin from alginate microspheres was studied in HCl pH 1.2 for 2 hours followed by PBS pH 7.4 by adding 10.6 grams Na_2HPO_4 , 1.5 gram KH_2PO_4 and 2 mL NaOH for about 6 hours at 100rpm and 37°C. 5 mL of samples were collected at time interval and was replaced by fresh medium.

The absorbances of ovalbumin were assayed using UV Vis spectrophotometry at λ max of 600 nm.

Table 1: Formula Ovalbumin-Alginate Microspheres

Cross Link Agent (M)	Aerosolisation			Drop Method		
	Alginate (%)			Alginate (%)		
	1	1.5	2.5	1	1.5	2.5
BaCl ₂	0.1					
	0.25					
	0.5					
	1.5					
CaCl ₂	0.1					
	0.25					
	0.5					
	1.5					

Results and Discussion

Morphology of ovalbumin-loaded alginate microspheres using aerosolisation technique: Morphology examinations of microspheres are shown in figure 1 and 2. From optical microscope,

it can be seen that microspheres produced using higher concentration of alginate (1 and 1.5%) and CaCl₂ (0.25; 0.5; 1.5M) resulted spherical microspheres, however the lowest concentration of CaCl₂ of 0.1M did not form microspheres instead gelling sheets (figure 1).

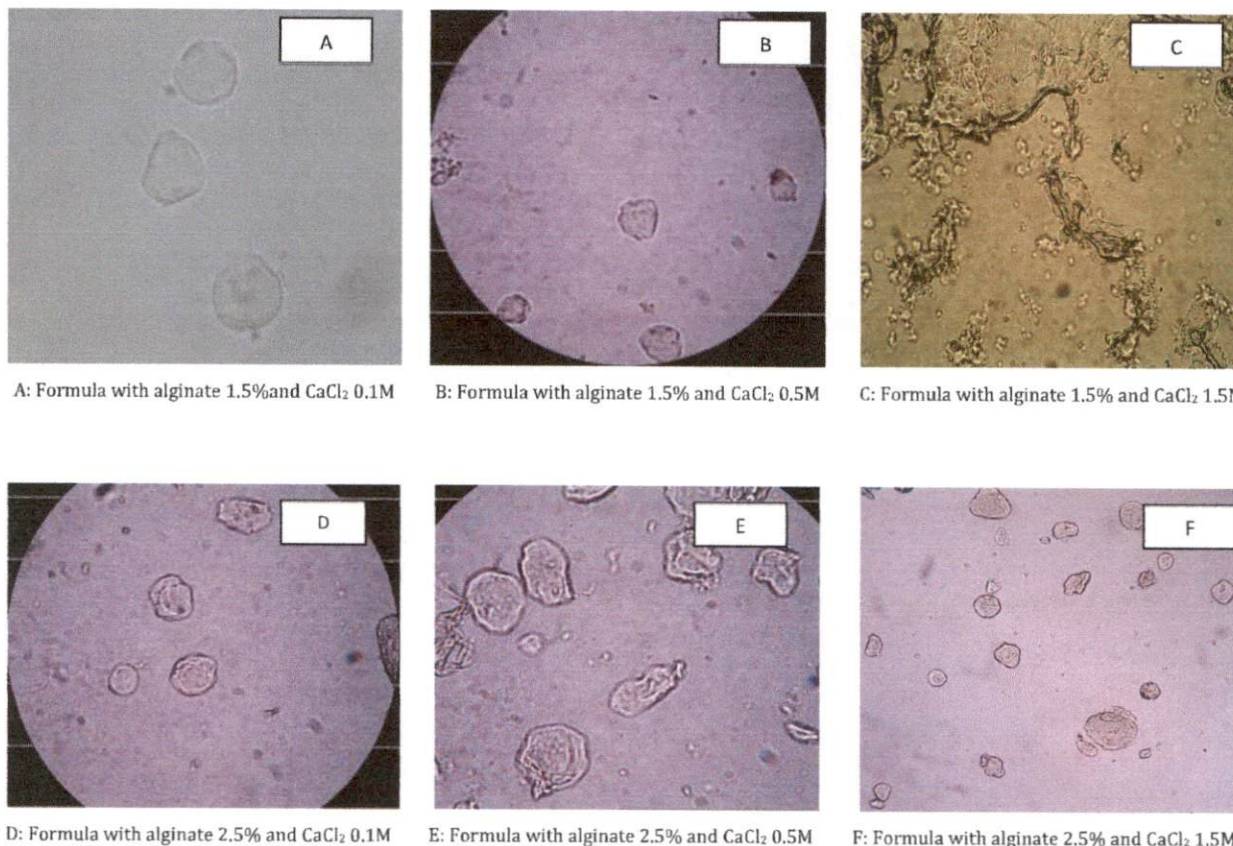


Fig. 1: Morphology of Ovalbumin-loaded Alginate Microspheres produced using aerosolization

In case of microspheres with BaCl₂ (0.1; 0.25; 0.5 dan 1.5M) crosslinker and alginate (1; 1.5 and 2.5%), all formulas formed almost spherical microspheres with some rough surfaces.

From SEM examination, smooth surface and almost spherical and regular microspheres were found (Figure 2), especially of the

formula with the highest CaCl₂ concentration (1.5M) in all alginate concentrations (1-2.5%). Additionally, Ba-alginate formed smoother, more spherical and smaller microspheres. This phenomenon was confirmed by whitest smooth freeze-dried ovalbumin-loaded Ba-alginate microspheres compared to more irregular and rough Ca-alginate microspheres.

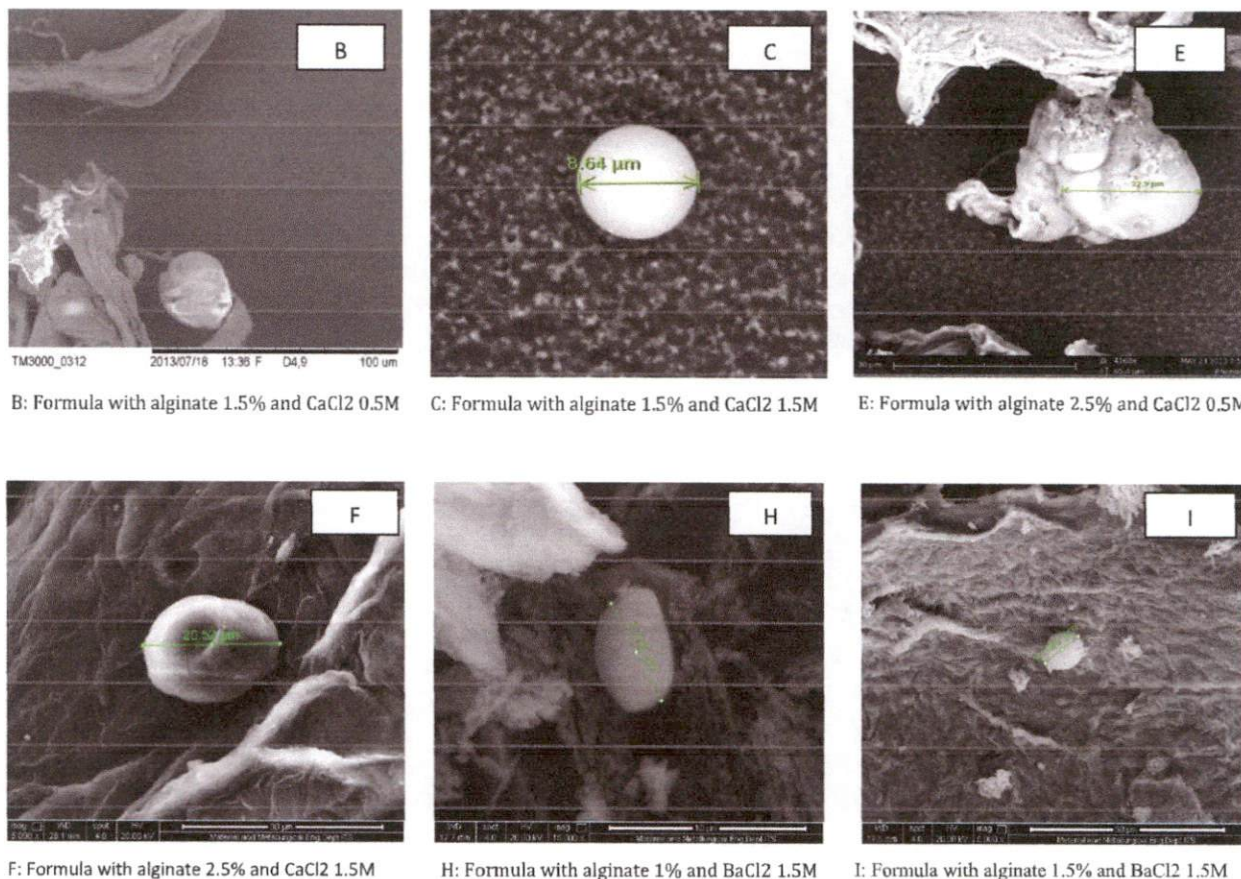


Fig. 2: SEM examination of Morphology of ovalbumin-loaded microspheres by aerosolization

Morphology of ovalbumin-loaded alginate microspheres using drop method

Drop method resulted much bigger microspheres either in hydrated and dried form as shown in figure 3 with almost smooth and nearly spherical microspheres.

Particle size

Particle size measurement of ovalbumin-loaded alginate microspheres produced using aerosolization with Ca²⁺ and Ba²⁺ crosslinker is shown in figure 4 and 5 and table 2.

Table 2: Particle size of ovalbumin-loaded alginate microspheres by drop method

Cross link agent (M)	Average Diameter (mm)			
	Alginate concentration (%)			
	1	1.5	2.5	
BaCl ₂	0.1 - 1.5M	1-2 mm	2-3 mm	2-3 mm
CaCl ₂	0.1 - 1.5M	1-2 mm	2-3 mm	2-3 mm

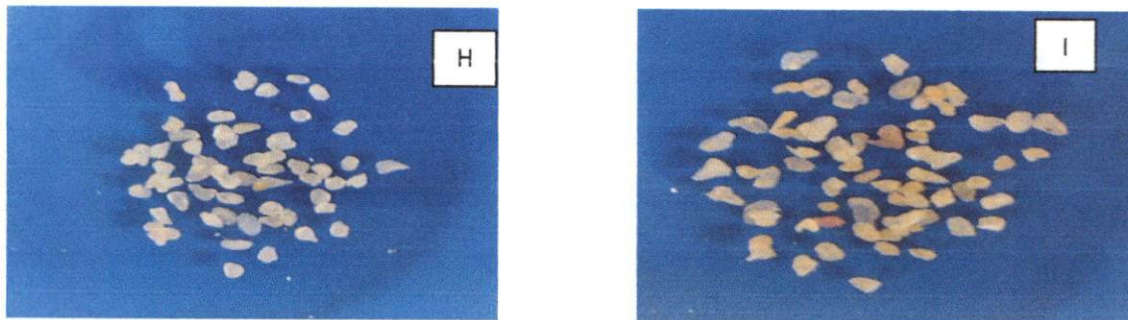


Fig. 3: Morphology of dried ovalbumin-loaded Ba-alginate microspheres

It was found that the average size of microspheres was between 12 and 30 μm from Ca and Ba-alginate microspheres produced using aerosolization (figure 4 dan 5). These was suitable for oral delivery application [10] and considering tha a good immune response was gained from microparticles having 1-30μm in size. The higher crosslinking agent's concentration decreased the particle size. This may due to an increase of Ca²⁺ tend to produce smaller and spherical microspheres [11]. This results was in agreement with study conducted by Joshi et al and Singh-Kumar [12,13]. In case of an increase in alginate concentration (from 1% to 2.5%), the particle size was increased, which may relate to its viscosity will may lead to produce bigger droplet size [11].

concentration, the entrapment efficiency was also increased [11,12,13].

Interestingly, encapsulation efficiency reduced up to 50% by increasing BaCl₂ (from 0.1 to 1.5M). This may be due to the excess of alginate amounts caused inhomogenous distribution of cross linking agent lead to a decrease in the encapsulation efficiency [15].

For protein loading, the higher CaCl₂ concentration or the higher alginate concentration, the higher loadings of ovalbumin. Again, this was because of the more availability of Ca²⁺ ions which crosslinked with carboxyl units formed gel network resulting the higher ovalbumin's content [14].

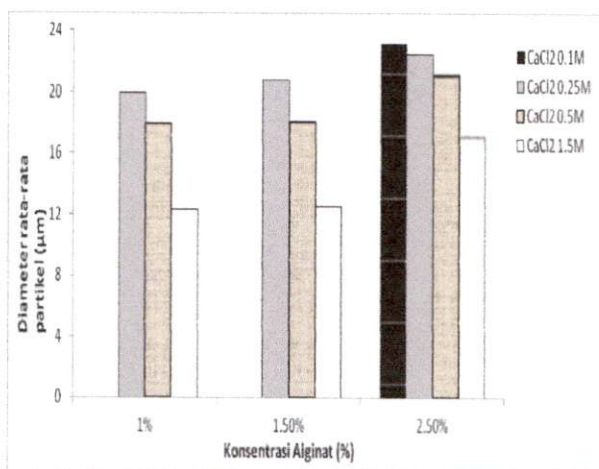


Fig. 4: Particle size of Ca-alginate microspheres by aerosolization

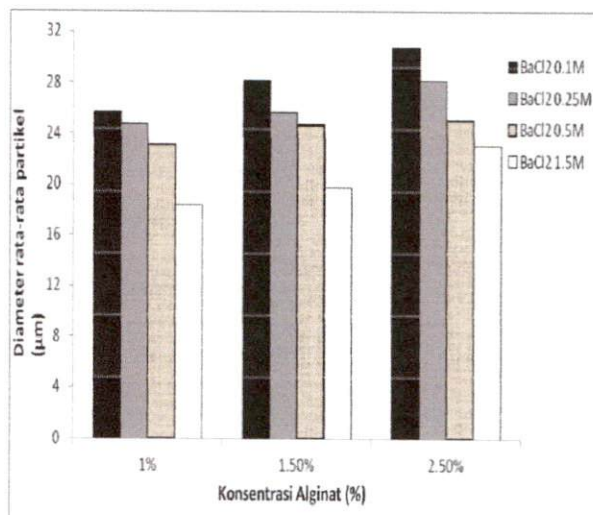


Fig. 5: Particle size of Ba-alginate microspheres by aerosolization

As predicted, the biggest size of microspheres of around 1 to 3 mm was produced by drop method in both Ba and Ca-alginate microspheres (table 2). This again may be because of their viscosity.

Encapsulation efficiency, loading and yield of microspheres

Encapsulation efficiency, protein loading and yield were determined in table 3. The percentages of entrapment are given in table 3 from which it can be concluded that in case of BaCl₂ with the increment of alginate concentration, the entrapment and loading increased except using alginate 2.5%. In contrast, when the highest concentration of alginate was used (2.5%), a decrease was occurred.

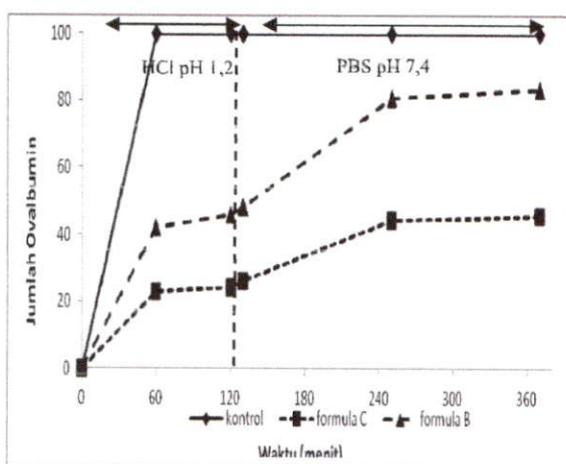
In terms of yield of microspheres produced using both CaCl₂ and BaCl₂, the yield increased by increasing cross linking agent concentration or alginate concentration, this findings were similar to study by Lin et al and Manjana et al. [11,16]. In all cases of drop method, it was difficult to determine encapsulation efficiency and yield of microspheres due to a problem in drying process. It was found that the drying process needs to be optimized for formula by this method. Aggregation phenomenon was occurred in hydrated stage of particles.

An increase of encapsulation efficiency was caused by more availability of Ca²⁺ ions crosslink with guluronic units of alginate providing more amounts of ovalbumin entrapped [14]. A similar phenomenon was also happened by increasing alginate

In vitro release study

Formula using 1.5% and 2.5% alginate with 1.5M CaCl₂ (formula B and C) were selected for in vitro release study with consideration of their highest encapsulation efficiency, loading and yield of

microspheres. Ovalbumin was used as a control. The in vitro release study was shown in figure 6.



Formula B : Formula with alginate 2.5% and CaCl₂ 1.5M
 Formula C : Formula with alginate 1.5% and CaCl₂ 1.5M

Fig. 6: Release profile of ovalbumin from formula microsphere B and C

It can be seen from figure 6 that the number of ovalbumin release from microspheres C was about 25% in acid pH for 2 hours incubation, much lower than than formula B (48,31%). After incubation in PBS pH 7.4, around 85% ovalbumin from formula B

was released in 370 minutes compared to formula C of 45%, this indicated that formula C was able to protect ovalbumin from acid pH and maintained its release in simulated intestine condition providing sustained release. This phenomenon was interested and could be attributed to an exposure of microspheres in acid condition followed by neutral condition may changed the egg-box structure between alginate and CaCl₂ causing an increase in ovalbumin release from formula B [17]. Another possibility was the highest of alginate concentration, the more viscous solution and this may cause penetration difficulty of alginate drop in to cross linking agent form optimum microspheres, which may produce less strong microspheres [18].

Based on that results, formula C was then selected to investigate more release behaviour by comparing with formula F (1.5% alginate and 0.5M CaCl₂ as shown in figure 7) to determine the effect of changing crosslinking concentration on the release of ovalbumin. In addition, formula C was also has smaller particles of around 12 µm. From figure 7 we can see that no significant differences of release of ovalbumin from formula C and F by using different concentration of CaCl₂. This may be because of a sudden swelling of microspheres in HCl pH 1.2 [19]. Compared to control, in all cases, release of ovalbumin from alginate microspheres was avoided and much slower than ovalbumin control. This was a good indication of the potential of ovalbumin-loaded alginate microspheres produced using ionotropic gelation with aerosolisation technique where only less than 40% of ovalbumin release in simulated gastric media and sustained release in simulated intestine media.

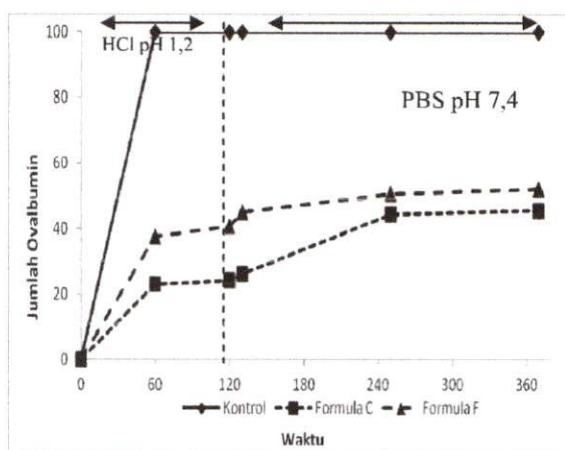
To study release behaviour of microspheres using BaCl₂, release study of formula microspheres with alginate 1.5% and 1.5M BaCl₂ (formula I) and 0.5M BaCl₂ (formula J) were compared (Figure 8).

Table 3: Encapsulation efficiency, protein loading and yield of microspheres using aerosolization

Cross link agent (M)	EE(%)	Protein loading (%)			Yield (%)					
		Conc Alginate (%)			Conc Alginate (%)					
		1	1.5	2.5	1	1.5	2.5			
BaCl ₂	0.1	31.22 ± 1.50	38.75 ± 2.10	70.32 ± 2.50	33.39 ± 3.20	33.47 ± 1.03	68.70 ± 2.21	25.20 ± 2.05	45.97 ± 1.80	51.18 ± 5.20
		38.56 ± 2.44	41.97 ± 3.25	62.79 ± 5.32	37.39 ± 1.55	43.52 ± 1.44	53.86 ± 3.04	31.52 ± 0.06	49.92 ± 10.22	58.20 ± 2.20
	0.25	52.65 ± 2.10	87.32 ± 1.50	40.79 ± 3.25	49.98 ± 2.10	80.76 ± 8.20	22.09 ± 1.86	49.47 ± 3.44	67.58 ± 6.20	92.8 ± 2.44
		59.45 ± 3.15	89.2 ± 1.05	35.28 ± 4.10	58.02 ± 5.12	89.49 ± 4.30	9.14 ± 2.15	73.18 ± 3.60	99.90 ± 0.15	92.8 ± 4.30
CaCl ₂	0.1	-	-	49.42 ± 8.22	-	-	49.58 ± 3.11	-	-	42.32 ± 7.28
		6.22 ± 0.20	5.04 ± 0.68	58.24 ± 2.35	18.12 ± 1.06	19.10 ± 2.20	68.73 ± 2.50	22.53 ± 2.86	33.60 ± 3.44	42.37 ± 2.26
	0.25	22.51 ± 2.26	38.31 ± 8.38	67.18 ± 8.03	18.76 ± 0.20	20.60 ± 5.78	70.10 ± 9.93	23.38 ± 0.13	33.56 ± 2.62	51.84 ± 6.29
		30.84 ± 0.60	63.75 ± 4.33	88.80 ± 0.52	36.00 ± 0.35	64.22 ± 8.82	74.50 ± 1.70	61.18 ± 3.26	62.30 ± 3.90	75.29 ± 8.56

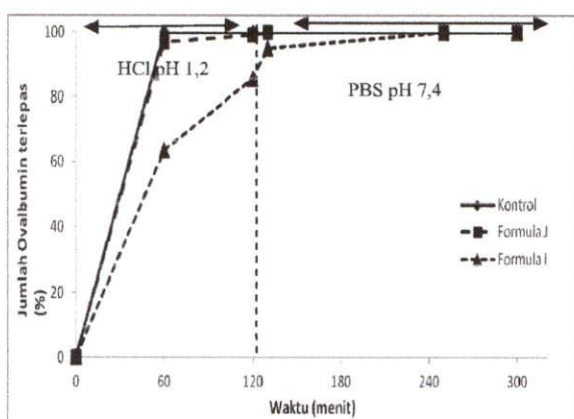
The burst release of ovalbumin was detected from microspheres formula I and J of about more than 60% during 1 hour incubation in HCl and nearly 100% ovalbumin release after 2 hours incubation in acid pH (formula I). This could be attributed to the inter-chain association between alginate polymer and Ba²⁺ can be temporary depending on the amount of barium content. The low level of barium content, temporary associations are obtained, as results, the more number of ovalbumin release from the microspheres [20]. Furthermore, the increase of ovalbumin from Ba-alginate microspheres compared to Ca-alginate microspheres were due to porosity of microspheres that form strong microspheres was not produced compared to Ca-alginate microspheres [16].

Another possible explanation was because of the cooling rate during freezing stage in the freeze-drying process exposed the Ba-alginate microspheres formed large hole and released ovalbumin faster (the shrinkage of microspheres was also indicated during SEM examination where size of microspheres were smaller than in hydrated stage). The smaller particle size, the larger surface areas, the more amounts of ovalbumin release in medium. It can be suggested for Ba-alginate microspheres, freeze-drying process was very critical step in order to produce a strong microspheres, therefore addition of lyoprotectant agent, protectant material which able to protect stability of protein during freezing and drying stage, are highly needed.



Formula C : Formula with alginate 1.5% and CaCl₂ 1.5M,
Formula F : Formula with alginate 1.5% and CaCl₂ 0.5M

Fig.7: Release profile of ovalbumin from formula microspheres C and F



Formula I : Formula with alginate 1.5% and BaCl₂ 1.5M
Formula J : Formula with alginate 1.5% and BaCl₂ 0.5M

Fig. 8: Release profile of ovalbumin from formula microspheres I and J

CONCLUSION

Small microspheres sizes (12-30µm) were successfully prepared by aerosolisation technique with BaCl₂ and CaCl₂ crosslinking agent. Several formulation parameters such as type and concentration of cross linking agents, concentration of polymer resulted significant influence on the size, morphology, encapsulation efficiency of ovalbumin, loading and yield microspheres. Ovalbumin-loaded alginate microspheres using alginate 1.5% and CaCl₂ 1.5M was found produced a sustained release, whereas microspheres using BaCl₂ need further investigation.

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DECLARATION OF INTEREST

The authors report no conflicts of interest.

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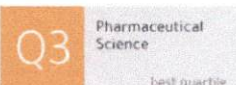
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