

# Preparation and characterization of alginate microspheres containing a model antigen

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## Abstract

The goal of this study was to evaluate the technological feasibility of delivering antigen using alginate microspheres. The microspheres were prepared by an emulsification technique and fully characterized as antigen delivery system. Selection of appropriate parameters enabled the preparation of alginate microspheres with a mean diameter of 8  $\mu\text{m}$ . The encapsulation efficiency of bovine serum albumin (BSA), chosen as model antigen, as well as the BSA loading were very high (> 90% and 10% w/w, respectively). The process of encapsulation did not affect the molecular weight or the antigenicity of the entrapped antigen. The in vitro release profile showed a fast release rate of encapsulated BSA, particularly in phosphate buffered saline solution. However, a decrease of the release rate was observed when alginate microspheres were coated with poly(L-lysine) or prepared with higher alginate molecular weight. Therefore, alginate microspheres appear, technologically, a promising antigen delivery system. © 1998 Elsevier Science B.V. All rights reserved.

*Keywords:* Alginate; Antigen; Bovine serum albumin; Characterization; Microspheres; Preparation

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## 1. Introduction

Since decade, efforts have been focused in the design of better antigen delivery systems which could increase mucosal and systemic immunity after oral (McGhee et al., 1992; Walker, 1994) or nasal administration (Almeida and Alpar, 1996;

Lemoine et al., 1998). Microencapsulation is the core technology in this endeavour for several reasons. Polymeric microspheres can protect the encapsulated antigen from acidic and proteolytic conditions. Moreover, they can be taken up by mucosa-associated lymphoid tissues, inducing immune responses to the encapsulated antigen. After oral administration, several authors (O'Hagan, 1996; Florence, 1997) demonstrated that the uptake of polymeric particles by the Peyer's patches

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M cells occur for microspheres having a diameter of 10  $\mu\text{m}$  or less. After nasal administration, Carr et al. (1996) showed that the uptake of polymeric particles by the nasal-associated lymphoid tissue (NALT) seems to occur, and Almeida et al. (1993) reported that latex particles penetrated into the blood circulation following intranasal administration. At least, due to the biodegradability of the polymeric matrix, the microspheres can control the release of antigen (McGhee et al., 1992; Walker, 1994).

In this context, the use of alginate microspheres as oral or nasal delivery system for antigen seems very attractive. First, the alginate matrix, which is formed by cross-linking the guluronic acid units with di- or trivalent metallic ions, e.g.  $\text{Ca}^{2+}$  (McDowell, 1977), could protect the antigen from hostile environments. Second, alginate possesses mucoadhesive properties (Junginger, 1991; Gombotz and Wee, 1998), which could increase the contact time between alginate microspheres and the absorptive epithelium and the mucosa-associated lymphoid tissue M cells (Bowersock et al., 1994; Critchley et al., 1994; Pereswetoff-Morath and Edman, 1995), and therefore could enhance the uptake of encapsulated antigen. Third, biodegradable alginate microspheres may show variable release kinetics as shown by numerous authors (Shiraishi et al., 1993; Machluf et al., 1996; Liu et al., 1997; Türkoglu et al., 1997). Fourth, the low toxicity and low immunogenicity of alginate (Lim and Sun, 1980; Gombotz and Wee, 1998) make this polymer a safe matrix. Fifth, alginate is readily available and inexpensive.

Recently, alginate microspheres have been used in several oral and nasal immunization studies (Wee et al., 1995; Bowersock et al., 1996; Suckow et al., 1996; Bowersock et al., 1998a,b; Cho et al., 1998). The nasal washes IgA levels and the serum IgG levels were increased after oral immunization (Bowersock et al., 1996; Suckow et al., 1996; Cho et al., 1998) and/or nasal immunization (Wee et al., 1995). However the preparation and particularly, the characterization of the alginate microspheres as antigen delivery system has not been extensively described in the literature.

Therefore, the goal of this study was to prepare and fully characterize alginate microspheres as antigen delivery system. The method of microsphere preparation was adapted from the method described by Wan et al. (1992), which allowed the preparation of alginate microspheres of 150  $\mu\text{m}$ . Different parameters influencing the alginate microsphere size were investigated in order to prepare microspheres having a mean diameter of 10  $\mu\text{m}$  or less, which could be taken up by the local lymphoid tissue M cells (O'Hagan, 1996; Florence, 1997). BSA was chosen as a water-soluble model antigen and the BSA loading and entrapment efficiency were determined. The effect of the microsphere preparative process on the molecular weight as well as on the antigenicity of encapsulated BSA were also analyzed. Finally, the release kinetic of BSA from alginate microspheres was studied.

## 2. Materials and methods

### 2.1. Materials

Sodium alginate (low or medium viscosity grade) was purchased from Sigma (St Louis, MO, USA). Sodium alginate solutions (5.0% w/v) of low or medium viscosity grade had viscosity at 20°C and 6.5  $\text{s}^{-1}$  shear force of 4.9 and 27.7  $\text{Pa} \cdot \text{s}$ , respectively, as determined with a Rheomat RM180 (Greifensee, Switzerland). The ratios of mannuronic acid to guluronic acid residues (M/G) were 1.6 for both viscosities. Hydroxypropylmethylcellulose (HPMC) and calcium chloride were obtained from Shin-Etsu (Tokyo, Japon) and Merck (Darmstadt, Germany), respectively. The surfactants used were sorbitan trioleate (Span 85), Federa (Brussels, Belgium); polyoxyethylene sorbitan trioleate (Tween 85), ICI (Essen, Germany); poly(vinylalcohol) (PVA) ( $M_w = 13000\text{--}23000$ ; 87.0–89.0% hydrolyzed), Aldrich Chemical (Bornem, Belgium), polyoxyethylene-polyoxypropylene copolymer (Pluronic F68), BASF (Brussels, Belgium) and sodium desoxycholate Fluka (Buchs, Switzerland). Iso-octane and isopropyl alcohol were from UCB (Braine L'Alleud, Belgium) and Across

Chimica (Kortrijk, Belgium), respectively. Bovine serum albumin (BSA) (fraction V), and poly(L-lysine) HBr ( $M_w$  1000–4000) (PLL) were provided by Sigma. Phosphate buffered saline solution (PBS) (pH 7.0;  $Ca^{2+}$ -,  $Mg^{2+}$ -free) was from GibcoBRL (Merelbeke, Belgium).

## 2.2. Methods

### 2.2.1. Alginate microsphere preparation

The ionic gelation process, which has been extensively used to prepare alginate beads (Kim and Lee, 1992; Shiraishi et al., 1993; Aslani and Kennedy, 1996; Hari et al., 1996), allows the preparation of large alginate microspheres ( $> 500 \mu\text{m}$ ) and could not be used to prepare small alginate microspheres (Türkoglu et al., 1997). The preparation method of alginate microspheres was thus adapted from an emulsification method described by Wan et al. (1992). Briefly, an aqueous solution containing sodium alginate and HPMC (9:1) (5.0% w/v) was dispersed in an iso-octane solution containing a lipophilic surfactant (Span 85) (2.0% w/w) by using a mechanical stirrer (Silverson L4R, Silverson Machines, Ltd, Bucks, England) at 8000 rpm. Microspheres prepared from a mixture of sodium alginate and HPMC have been found more spherical and showed smoother surfaces than those prepared with sodium alginate alone (Wan et al., 1992). In the case of BSA loaded microspheres, 1 ml of BSA solution (2.5; 5.0; 10.0 or 15.0% w/v) was added in the aqueous solution containing sodium alginate (2.2; 4.4; 8.8; 13.3% w/w BSA/alginate). An aqueous solution containing an hydrophilic surfactant was then added and the emulsion was stirred for 15 min. A calcium chloride solution (8.0% w/v) was added and the dispersion was mixed for another 10 min. Isopropyl alcohol was then used to further harden the formed microspheres. The microspheres were collected by filtration, washed three times with isopropyl alcohol and finally dried 2 h at 37°C.

In order to prepare alginate microspheres having a diameter of 10  $\mu\text{m}$  or less, the effects of various operational and formulation factors on the microsphere size were investigated. The alginate concentration (1.0, 2.5 or 5.0% w/v), the hydrophilic surfactant nature (sodium desoxycholate, PVA,

Pluronic F68 or Tween 85), the alginate molecular weight (low or medium viscosity grade), or the influence of sonication were studied. For each variable studied, batches of microspheres were prepared in duplicate.

### 2.2.2. Morphology analysis and size determination of alginate microspheres

The shape of the microspheres was characterized by optical microscopy (Carl Zeiss, Oberkochen, FRG). The volume mean diameters of the microspheres were determined in ultrapure water (Sation 9000, Sation, Barcelona, Spain) by laser diffraction (Fraunhofer model) (Coulter LS 230, Hiialeah, Florida, USA) and measured in triplicate for each batch. The evolution of the volume mean diameters were also evaluated at 0, 30 min, 1 h, 2 h, 3 h and 4 h after resuspension of the alginate microspheres in water.

### 2.2.3. BSA entrapment in alginate microspheres

To evaluate the BSA entrapment efficiency in alginate microspheres, known amount of BSA loaded microspheres were accurately weighted and dissolved in NaOH 5 N for 2.5 h. The pH was adjusted to 11.5, using HCl 5 N. The samples were centrifuged at  $560 \times g$  for 10 min to eliminate non soluble residuals and a bicinchoninic acid protein assay (BCA) (Smith et al., 1985) was used to determine the BSA concentration in the supernatants. From these results, the percentage (w/w) of BSA entrapped per dry weight of microspheres (BSA loading) and the ratio [actual BSA content (w/w)/theoretical BSA content (w/w)]  $\times 100$  (encapsulation efficiency) were determined as previously described (Jeffery et al., 1993). For each BSA concentration, the microsphere preparation as well as the dosage of encapsulated BSA were realized in triplicate.

### 2.2.4. Molecular weight and antigenicity of encapsulated BSA

**2.2.4.1. Polyacrylamide gel electrophoresis (PAGE).** The molecular weight integrity of encapsulated BSA was determined by polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970). Encapsu-

lated BSA released from BSA loaded alginate microspheres (BSA loading = 6.3% w/w) in PBS, native BSA and a molecular weight reference marker (molecular weight 25–175 kDa) were loaded onto 10% acrylamide gel and run using Bio-Rad Mini Protean II Electrophoresis System. Proteins were visualized by Coomassie blue staining.

#### 2.2.4.2. Western-blot

The antigenicity of entrapped BSA was assessed by Western blotting (Towbin et al., 1979). BSA samples were transferred from the acrylamide gel onto the nitrocellulose membrane using Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell. The blot was then incubated with a mice monoclonal anti-bovine serum albumin (Sigma Bio-Sciences, St Louis, MO, USA). After washing, the blot was incubated with a rabbit anti-Mouse IgG (H et L) conjugated to phosphatase alkaline (Promega, Madison, WI, USA). The ability of the antiserum to recognise BSA was demonstrated colorimetrically using nitro blue tetrazolinum (NBT) and 5-bromo-4-chloro-3-indolyl-1-phosphate (BCIP) (Promega, Leiden, The Netherlands) in Tris buffer pH 9.5.

#### 2.2.5. *In vitro* release study of BSA from alginate microspheres

The *in vitro* release study of BSA from BSA loaded alginate microspheres (BSA loading = 6.3% w/w) was carried out in PBS as well as in ultrapure water (Sation 9000, Sation, Barcelona, Spain), in order to better understand the mechanisms of antigen release through alginate microspheres. The influence of the concentration of PLL used for coating microspheres as well as the influence of the alginate molecular weight (low or medium viscosity grade) on the release profile of BSA were also investigated. The PLL coating were formed on the surface of the harvested microspheres by suspending them into a solution of PLL (0.05, 0.25 or 1.00% w/v), for 10 min. The suspensions were then centrifuged at  $560 \times g$  for 7 min and the supernatants eliminated.

For the *in vitro* release, accurately weighted amounts (15 mg) of alginate microspheres were placed into test tubes containing 1 ml of ultrapure

water or PBS. The tubes were then incubated at 37°C under continuous shaking. At selected time intervals, tubes were collected and centrifuged at  $560 \times g$  for 7 min. The BSA concentrations in the supernatants were determined by the BCA assay (Smith et al., 1985). Each *in vitro* release study was performed in triplicate. Since we did not know the exact time required for the coating be formed on the surface of the microspheres and since, in absence of coating, the burst effects were high and fast, the amount of BSA released during the PLL coating was dosed in the supernatant and added to the amount of BSA released at the selected time intervals of the *in vitro* release. This could avoid to give the impression that the PLL coating decrease the release rate of BSA due to the burst effect happening during the PLL coating and not to the coating itself.

### 3. Results and discussion

#### 3.1. Morphology analysis and size determination of alginate microspheres

Calcium alginate microspheres were prepared by an emulsification method adapted from the method of Wan et al. (1992). These authors showed that their method allowed the preparation of alginate microspheres smaller than 150  $\mu\text{m}$ . In order to prepare much smaller alginate microspheres ( $< 10 \mu\text{m}$ ), the effect of formulation variables (alginate concentration, surfactant nature, alginate molecular weight and sonication) on alginate microsphere preparation (general aspect, microsphere size) were investigated (Table 1, Fig. 1A-1C).

The influence of alginate concentration was evaluated with three different concentrations (1.0; 2.5; 5.0%) (Table 1). As expected, decreasing the alginate concentration decreased the microsphere size (Jeffery et al., 1991). However, high degree of clumping of the small microspheres was observed. This could explain why Pepeljnjak et al. (1994) obtained higher size of alginate microspheres when the microspheres were prepared with lower concentration of alginate. Therefore, an alginate concentration of 5% was used in the further preparations.

Table 1  
Morphology analysis and size determination of alginate microspheres

Factors studied		General aspect (optical microscope)	Estimated size of microspheres (optical microscope)	Volume mean diameter $\pm$ S.E.M. <sup>c</sup> (Coulter LS230)
Alginate concentration <sup>a</sup>	1.0%	Small microspheres aggregates: + + +	< 1 $\mu$ m	ND
	2.5%	Small microspheres aggregates: + + +	< 10 $\mu$ m	ND
	5.0%	Large microspheres aggregates: +	5–90 $\mu$ m	70.9 $\pm$ 0.7
Surfactant nature <sup>b</sup>	Sodium desoxycholate	Very heterogeneous aggregates: + + +	1–150 $\mu$ m	ND
	PVA	Very heterogeneous aggregates: +	1–130 $\mu$ m	ND
	Pluronic F68	Very heterogeneous aggregates: + + +	1–130 $\mu$ m	ND
	Tween 85	Less heterogeneous aggregates: +	5–90 $\mu$ m	70.9 $\pm$ 0.7
Alginate molecular weight <sup>c</sup>	Low viscosity grade	Small microspheres aggregates: +	5–20 $\mu$ m	11.9 $\pm$ 0.1
	High viscosity grade	Large microspheres aggregates: +	5–90 $\mu$ m	70.9 $\pm$ 0.7
Sonication <sup>d</sup>	No sonication	Small microspheres aggregates: +	5–20 $\mu$ m	11.9 $\pm$ 0.1
	Sonication	Small microspheres aggregates: 0	5–20 $\mu$ m	7.6 $\pm$ 0.1

<sup>a</sup> Tween 85, alginate high viscosity grade, no sonication.

<sup>b</sup> Alginate 5%, alginate high viscosity grade, no sonication.

<sup>c</sup> Alginate 5%, Tween 85, no sonication.

<sup>d</sup> Alginate 5%, Tween 85, alginate low viscosity grade.

<sup>e</sup> For each variable studied, batches of microspheres were prepared in duplicate. For each batch, the volume mean diameter was measured in triplicate. The mean and S.E.M. of the six measures were then calculated.

+ + + > 60% aggregated microspheres.

+ + 30–60% aggregated microspheres.

+ 1–30% aggregated microspheres.

0 < 1% aggregated microspheres

ND, not determined.

As the present method of microsphere preparation involves emulsification, the surfactants could play an important role in the microsphere production (Jeffery et al., 1993). Therefore, the influence of the type of surfactant was evaluated using four surfactants, namely sodium desoxycholate, PVA, Pluronic F68 and Tween 85 (Table 1). Tween 85 produced less heterogeneous and smaller microspheres as compared to sodium desoxycholate, PVA or Pluronic F68. This could be due to the variation of the hydrophilic-lipophilic balance as well as to the nature of the surfactant as shown

by Wan et al. (1994).

The influence of the alginate molecular weight on the final product was evaluated with alginate of low viscosity and medium viscosity grade (Table 1). It appears that alginate molecular weight has an important effect on the alginate microsphere size, since low viscosity grade alginate provided much smaller microspheres (11.9  $\pm$  0.1  $\mu$ m) as compared to medium viscosity grade alginate (70.9  $\pm$  0.7  $\mu$ m) (Table 1, Fig. 1A and B). Increasing the alginate molecular weight could extend the total number of cross-linking between

the guluronic acid units and the calcium, and therefore the microsphere size as also showed by Payne et al. (1995). The microsphere size re-

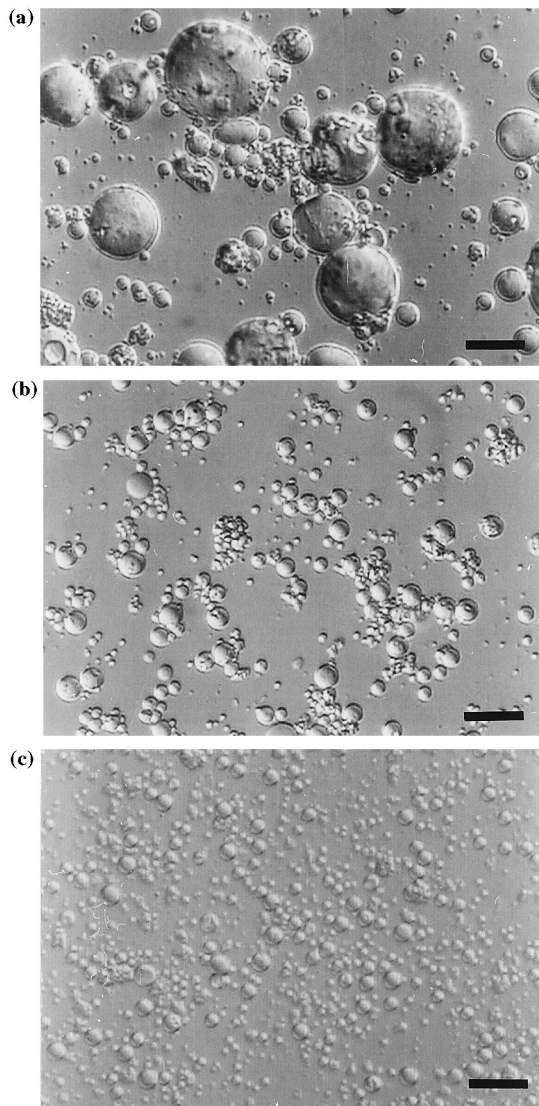


Fig. 1. (A) Alginate microspheres prepared with alginate of medium viscosity grade. The microspheres are large (volume mean diameter =  $70.9 \pm 0.7 \mu\text{m}$ ) and some microspheres are aggregated. Scale bar  $62.5 \mu\text{m}$ . (B) Alginate microspheres prepared with alginate of low viscosity grade. The microspheres are small (volume mean diameter =  $11.9 \pm 0.1 \mu\text{m}$ ) and some microspheres are aggregated. Scale bar  $62.5 \mu\text{m}$ . (C) Alginate microspheres prepared with alginate of low viscosity grade and sonicated. The microspheres are small (volume mean diameter =  $7.6 \pm 0.1 \mu\text{m}$ ), spherical and there is no evidence of aggregation. Scale bar  $62.5 \mu\text{m}$ .

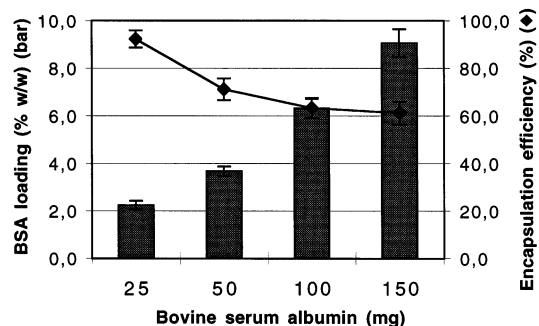


Fig. 2. BSA loading (bar) and encapsulation efficiency (◆) across the initial amount of BSA.

maintained stable in ultrapure water for at least 4 h, since the microsphere size was, after resuspension of the particles in water during 0 min, 30 min, 1 h, 2 h, 3 h and 4 h of 11.8, 12.0, 12.1, 11.3, 11.4 and 11.7  $\mu\text{m}$ , respectively. In addition, sonication of microspheres prepared with low viscosity grade alginate allowed to reduce clumping and therefore the microsphere size ( $7.6 \pm 0.1 \mu\text{m}$ ) (Table 1, Fig. 1C). Therefore, excepted when mentioned, alginate microspheres were prepared with an initial alginate concentration of 5% w/w, Tween 85 and low molecular weight alginate in the next experiments.

### 3.2. BSA entrapment in alginate microspheres

To assess the ability of alginate microspheres to encapsulate proteins, the influence of the BSA initial amount on BSA loading and encapsulation efficiency was studied. As shown by Fig. 2, the encapsulation efficiency was very high for the four amounts of BSA studied, ranging from  $61.0 \pm 4.8$  to  $92.0 \pm 3.6\%$ . Increasing the BSA initial amount increased the BSA loading ( $2.2 \pm 0.2$ – $9.1 \pm 0.6\%$  w/w), but decreased the encapsulation efficiency ( $92.0 \pm 3.6$ – $61.0 \pm 4.8\%$ ).

The high encapsulation efficiency can be easily explained by the method of microsphere preparation. An aqueous sodium alginate solution containing the BSA was dispersed in an organic phase to form a water-in-oil emulsion (Wan et al., 1992).  $\text{CaCl}_2$  was then added and probably merged with the internal aqueous phase of alginate. The alginate droplets formed gel spheres

instantaneously, entrapping the BSA in a three-dimensional lattice of ionically crosslinked alginate. Increasing the initial amount of BSA in the aqueous solution of alginate increased the amount of BSA in the lattice and therefore the BSA loading. However, the encapsulation efficiency was decreased suggesting that the quantity of polymer present becomes insufficient to entrap the total amount of protein.

Other authors also reported high encapsulation efficiencies for macromolecular drugs in alginate microspheres or beads, e.g. 95.0% for BSA-fluorescein isothiocyanate (Liu et al., 1997); 80.0% for blue dextran (Kim and Lee, 1992). Lower encapsulation efficiencies were generally obtained for low molecular weight drugs, e.g. 4.0% for nitrofurantoin (Hari et al., 1996), 32% for indomethacin (Shiraishi et al., 1993). The porosity of alginate beads and microspheres (Pfister et al., 1986; Liu et al., 1997; Türkoglu et al., 1997) could be responsible of the fast release of small drugs (i.e. during microspheres washes) and could explain the low encapsulation efficiencies. Moreover, the encapsulation efficiencies of water soluble drugs are in general lower than for slightly soluble or insoluble drug (Aslani and Kennedy, 1996; Shiraishi et al., 1993). Wan et al. (1992) and Hari et al. (1996) also showed that increasing the amount of drug in the sodium alginate aqueous phase increased the drug content.

### 3.3. Molecular weight and antigenicity of encapsulated BSA

A critical point in developing a carrier system for antigens is the preservation of their molecular weight and particularly their antigenicity. During the alginate microsphere preparation, BSA was exposed to potentially harsh conditions, such as shear force or contact with surfactants and organic solvents. This may result in alteration of the molecular weight and decrease of the antigenicity of the protein. Therefore the molecular weight of BSA was evaluated by PAGE (Fig. 3) and its antigenicity by Western-blot (Fig. 4). In both cases, identical bands were observed for the native BSA and the BSA released from alginate microspheres. Hence the data suggest that the molecu-

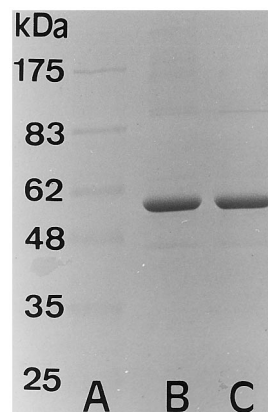


Fig. 3. Polyacrylamide gel electrophoresis of BSA released from BSA loaded alginate microspheres. Lanes represent the molecular weight marker (A), native BSA (B) and BSA released from alginate microspheres (C).

lar weight of BSA was not affected by the entrapment procedure (Fig. 3) and that an anti-serum raised against non-entrapped BSA still recognized encapsulated BSA (preservation of this epitope) (Fig. 4). Alginate microspheres have been used in several recent oral and nasal immunization studies (Wee et al., 1995; Bowersock et al., 1996; Suckow et al., 1996; Bowersock et al., 1998a,b; Cho et al., 1998). However the integrity of molecular weight and antigenicity of encapsulated antigens have never been reported in the literature.

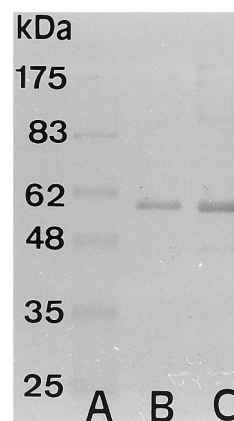


Fig. 4. Western-blot of BSA released from BSA loaded alginate microspheres. Lanes represent the molecular weight marker (A), native BSA (B) and BSA released from alginate microspheres (C).

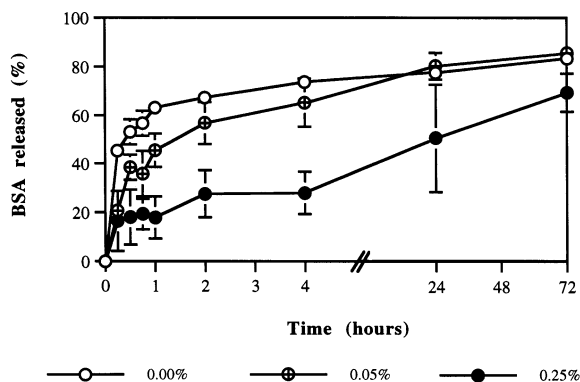


Fig. 5. Release profiles of BSA in ultrapure water, from BSA loaded alginate microspheres prepared with low molecular weight alginate and coated with various concentrations of poly(L-lysine).

### 3.4. *In vitro* release studies of BSA from alginate microspheres

In order for the microencapsulated antigen to elicit an immune response in the local lymphoid tissues, the antigen must be released from microspheres. Therefore the release profiles of BSA from alginate microspheres were evaluated in ultrapure water as well as in PBS.

In ultrapure water (Fig. 5), the release profile was characterized by an important initial burst effect ( $\cong 45\%$ ), followed by a continuous and fast release phase (time required for 50% release of BSA = 23 min). The highly porous structure of alginate microspheres (Pfister et al., 1986; Liu et al., 1997; Türkoglu et al., 1997), formed by gelation of sodium alginate with  $\text{Ca}^{2+}$ , could explain this fast release pattern of encapsulated BSA. In order to delay the BSA release, the microspheres were coated with various PLL concentrations (Fig. 5). Both the burst effect and the continuous release phase appeared highly dependent on the concentration of PLL used for the coating. The burst effects decreased to  $\cong 35\%$  and  $\cong 16\%$  and the times required for 50% release of BSA increased to 84 min and 24 h, for 0.05 and 0.25% PLL coating, respectively. The positively charged PLL, which can build ionic linkages with the surface of the negatively charged alginate microspheres and therefore form a semi-permeable

membrane on the outside of the microspheres (Coromili and Chang, 1993; Payne et al., 1995; Thu et al., 1996), could explain the decrease in both the burst effect and the release rate in presence of PLL coating.

In PBS (Fig. 6), the release profiles were characterized by very large initial burst effects ( $\cong 80$ ,  $\cong 63$  and  $\cong 59\%$ ) and short times required for 50% release (9, 12 and 13 min) for 0.00, 0.05 and 0.25% PLL coating, respectively. As compared to ultrapure water, faster release rates were obtained in PBS, and BSA was released very quickly. These phenomenae could be attributed to the removal of the cross-linker bivalent cation, calcium, from the alginate microspheres by monovalent cations, such as sodium or potassium contained in PBS. These ion exchanges could cause the erosion of the microspheres and therefore the fast release of BSA. Moreover, the influence of the PLL coating was less evident. Increasing the PLL concentration or the reaction time between the microspheres and the PLL solution, as well as decreasing the PLL molecular weight could decrease the burst effect and the release rate of the antigen in PBS (Machluf et al., 1996).

Other authors also reported important burst effects and fast release rates of drugs from alginate beads or microspheres (Kim and Lee, 1992; Murata et al., 1993; Aslani and Kennedy, 1996;

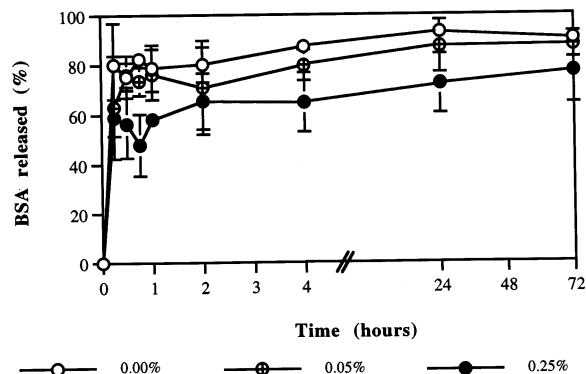


Fig. 6. Release profiles of BSA in phosphate buffered saline, from BSA loaded alginate microspheres prepared with low molecular weight alginate and coated with various concentrations of poly(L-lysine).



Hari et al., 1996; Kikuchi et al., 1997; Liu et al., 1997; Türkoglu et al., 1997). Moreover, Aslani and Kennedy (1996) and Türkoglu et al. (1997) also showed that the release of drugs were slower in ultrapure water than in PBS, and Machluf et al. (1996) that the release of encapsulated liposomes was decreased by increasing the PLL coating concentration.

Two processes can explain the release of a drug from a particle: diffusion and erosion. BSA could diffuse out of the alginate microspheres, following the water phase that fills the matrix of the microspheres. BSA could also be released from the alginate microspheres through the erosion of the matrix. Erosion could occur through the reversal of the gelation reaction, thus resulting in the solubilization of alginate molecules, or through the degradation of the alginate backbone into smaller molecular weight components. In ultrapure water, the erosion process is not predominant because alginate is stable in aqueous ultrapure environment at 37°C (Vandenbossche and Remon, 1993), and the reversal of the gelation reaction does not occur. Therefore, in ultrapure water, the process of BSA release was mainly controlled by the diffusion process as also showed by other authors (Kim and Lee, 1992; Türkoglu et al., 1997). In PBS, in addition to the diffusion process, the ion exchanges cause the erosion of the microspheres, which greatly increases the BSA release rate (Türkoglu et al., 1997).

The influence of the alginate viscosity grade on the BSA release from alginate microspheres was also investigated in water and in PBS (Fig. 7). As the molecular weight of the alginate increased, a marked decrease of the burst effect and of the release rate was observed, both in ultrapure water and in PBS. In water, the burst effects were  $\cong 45\%$  and  $\cong 11\%$  and the times required for 50% release were 24 min and 99 h, for the low and the medium viscosity grade, respectively. In PBS, the burst effects were  $\cong 80\%$  and  $\cong 54\%$  and the times required for 50% release were 9 and 70 min, for the low and the medium viscosity grade, respectively. Murata et al. (1993) also showed that the release rate of blue dextran from alginate beads prepared with low molecular weight alginate was faster than from those prepared with high molecular weight

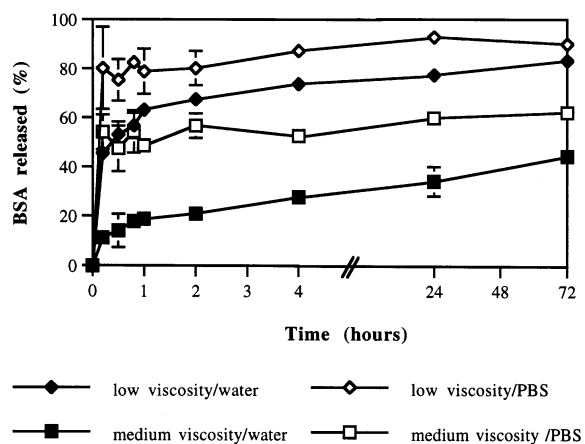


Fig. 7. Release profiles of BSA in ultrapure water or in phosphate buffered saline, from BSA loaded alginate microspheres prepared with alginate of low or medium viscosity grade.

alginate. This slower release rates for high molecular weight alginate could be explained by several factors. First, high molecular weight alginate could produce microsphere matrices of higher density (Pepeljnjak et al., 1994). Second, the number of crosslinking points could be increased (Kikuchi et al., 1997). Third, increasing the alginate molecular weight also increased the microsphere size (Fig. 1A) and therefore increased the time of BSA diffusion and/or the time of alginate microsphere erosion, as also shown by Kikuchi et al. (1997).

Therefore, the antigen release kinetics can be controlled by regulating the PLL coating concentration as well as the alginate molecular weight. However, increasing the alginate molecular weight also increased the microsphere size, which could prevent the microsphere uptake by the local lymphoid tissue M cells.

#### 4. Conclusion

This study demonstrated that spherical and non aggregated alginate microspheres with a mean diameter of 8  $\mu\text{m}$  can be prepared by an emulsification method. High encapsulation efficiency of BSA ( $> 90\%$ ) and high BSA loading (10% w/w) were achieved. Neither the molecular weight, nor the antigenicity of encapsulated BSA were af-

fectured by the entrapment procedure. The release of BSA from alginate microspheres was quite fast, particularly in PBS. However, it was possible to delay the release of BSA, particularly by coating the alginate microspheres with PLL. Therefore, alginate microspheres appear, technologically, a promising delivery system for antigen.

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