Production and characterisation of alginate microparticles incorporating *Aeromonas hydrophila* designed for fish oral vaccination

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Abstract

Oral vaccination is a relevant alternative for fish immunisation in intensive culture. However, its effectiveness is limited by possible vaccine degradation in the fish digestive system. The purpose of this work was to obtain stable biocompatible alginate microparticles entrapping inactive *Aeromonas hydrophila* cells for fish oral immunisation. The particles were prepared through an emulsion-based methodology, employing different vegetable oils containing the surfactant Span 80, varying the ratio between aqueous alginate and oil phases, the stirring rate and the initial concentration of *A. hydrophila*. Microparticles stable under gastrointestinal conditions presenting mean diameters lower than 50 μm were obtained with 1:9 (v/v) of aqueous phase containing 3.5% alginate (w/v) in relation to the oil phase (corn oil with 0.2% v/v Span 80) under stirring at 2000 rpm, with bacterium encapsulation efficiencies up to 100%.

Keywords: Microparticles; Alginate; Fish vaccine; Encapsulation; Emulsion; *Aeromonas hydrophila*

1. Introduction

Aquacultural output should be increased several fold to meet the rising demands for fish in the near future [1]. In tropical and subtropical inland fisheries, several fish species, such as Nile tilapia (*Oreochromis niloticus*), present high growth rates, good productivity per unit volume of water as well as economically efficient feed conversion. One of the difficulties of intensive fish culture is the control of diseases caused by pathogens such as *Aeromonas hydrophila*, one of the most common bacteria in freshwater. Possible consequences of *A. hydrophila* infection to fish are skin lesions, which can result in haemorrhagic septicemia, followed by high mortalities [2]. Then, control actions against *A. hydrophila* should be strongly considered, mainly when intensive fish culture is performed.

Fish losses caused by diseases and the use of antibiotics can be significantly reduced in the aquaculture industry if appropriate strategies are adopted, such as immuno prophylaxis. According to Gudding et al. [3], stimulation of specific and non-specific immunity is the basis for developing aquaculture into sustainable bioproduction in aquatic ecosystems.

Oral vaccination has been considered a relevant alternative for fish immunisation, in spite of yielding the lowest immunity when compared to injection, immersion or spraying routes [4]. The advantages of oral vaccine delivery in fish include easy of application, improved safety, substantial reduction on the stress imposed to fish by the immunological agent parenteral administration, as well as the possibility of rapid vaccination of a large number of animals with reduced costs [4,5]. However, the effectiveness of this approach is limited, since the vaccine can be degraded in the fish digestive system [3] and may also interact with feed components. Therefore, the encapsulation of immunising agents in polymeric devices such as microparticles constitutes a relevant strategy for effective oral antigen delivery to fish.

Different approaches have been considered concerning to the incorporation of fish immunising agents in microparticles [5–10]. However, it should be pointed out that the mechanisms involved in antigen uptake and presentation after fish oral vaccination are not fully understood. Therefore, the selection of the appropriate characteristics of the particles designed to...
perform the desired task is not straightforward. In comparison with mammalian vaccination, biocompatible particles smaller than 10 μm in diameter loaded with high concentration of the antigenic agent and stable in gastrointestinal conditions would be desired [11–13]. On the other hand, if only the protection of the antigenic agent from the gastric acidic conditions and from proteolysis is aimed, particles larger than 10 μm (diameters up to 300 μm) could be successfully employed [11]. According to the work recently published by Romalde et al. [10], a spray-dried formulation consisting of 30 μm in diameter alginate microparticles incorporating *Lactococcus garvieae* rendered significant levels of protection to rainbow trout (*Oncorhyncus mykiss*) after oral administration.

Alginate is one of the most widely used carriers for the controlled release of different types of active agents. Among many interesting characteristics which turns alginate very attractive as a constituent of delivery systems, its mucoadhesive properties, stability to proteolytic and acid conditions, biodegradability, low toxicity, availability and relatively low cost when compared to other hydrogel matrixes can be pointed out [11,12,14]. Moreover, mild gel formation conditions are involved, consisting of cross-linking the guluronic acid units from alginate with divalent metallic ions such as calcium.

Among the several factors which can affect the characteristics of alginate microparticles such as their average diameter, size distribution and antigenic agent incorporation efficiency, some of the most relevant are: microsphere production technique, alginate type and concentration, antigenic agent concentration (e.g. inactive cells), as well as the nature and composition of the gelification agent [11,15–18]. When particle formation through emulsion followed by ionic gelation approaches are employed, the choice of equipment design, stirring rate [16], temperature, emulsifying agent type and concentration [19], as well as the nature and proportion of aqueous and non-aqueous phases will certainly affect particle characteristics. Unfortunately, details on alginate microparticle production methodology are sometimes not fully provided, rendering difficulties in the reproduction of previously published results.

The purpose of this work was, therefore, to systematically study the preparation of microparticles encapsulating inactive *A. hydrophila* cells designed for fish oral immunisation. In order to be effective as oral bacterin (a vaccine constituted of inactivated bacteria) delivery agents to fish, biocompatible alginate particles containing appropriated amounts of *A. hydrophila*, smaller than 50 μm in diameter and stable to pH values in the range of 2–9 were aimed. Among other reasons, alginate was selected as the structural constituent of the microparticles since it may, by itself, induce immune response against bacterial infection in fish [20].

## 2. Materials and methods

### 2.1. Materials

Low viscosity sodium alginate (Sigma Chemical Co., USA), Span 80 (Fluka ChemiKa, Switzerland) and different vegetable oils (corn, sunflower, soyabean or canola, from Liza brand, Brazil, purchased in a local supermarket) were employed for particle preparation. The bacterium *A. hydrophila* was obtained from the American Type Culture Collection (ATCC 7966), USA. All other reagents used were, at least, of analytical grade.

### 2.2. Preparation of the bacterin

The selected strain of *A. hydrophila* was cultivated for 24 h at 30°C in triptose soybean agar (TSA, Biolife, Italy) medium. The cells were then inoculated in three 500 mL Erlenmeyer flasks containing each 250 mL of brain–heart broth (BHI, Difco, USA) and incubated for 24 h at 30°C in a shaker at 150 rpm. After this period, the culture was inactivated by the addition of formalin, to achieve a concentration of 0.5% (v/v), and stored at 4°C for 24 h. To ensure cell inactivation, an aliquot of the bacteria was inoculated in TSA and incubated at 30°C for 24 h. The inactive cells were then centrifuged at 3000 rpm for 15 min and the cell pellet was resuspended in phosphate buffered saline (PBS) at pH 7.2. Cells were stored in this buffer at 4°C for at most 2 weeks. Before incorporation in the alginate particles, the cells were centrifuged at 10,000 rpm for 10 min at 6°C, washed three times and resuspended in saline (NaCl at 0.9% m/v) at pH 7.0, resulting in cell suspensions containing from 40 to 60 mg of cells (in terms of dry weight) per millilitre.

### 2.3. Particle production

The particles were prepared using the emulsion formation approach based on the procedure of Mofidi et al. [16], roughly as illustrated in Fig. 1. Briefly, aliquots of 7–35 mL of aqueous solutions containing 2–5% (m/v) of sodium alginate were mixed with 35–63 mL of vegetable oil containing from 0.2 to 2.0% (v/v) Span 80 at stirring rates varying from 500 to 2000 rpm for 10 min. For bacteria incorporation, from 16.6 to 99.6 mg of cells (in terms of dry weight) suspended in saline were mixed with alginate aqueous solution, totalising 7 mL of a 3.5% polysaccharide suspension that was then added to the vegetable oil containing Span 80. The obtained emulsion was mixed with 15.5 or 70 mL of a gelification solution (containing 5 g CaCl2·2H2O dissolved in 37.5 mL distilled water, 37.5 mL ethanol and 2.5 mL acetic acid), for 10 min at 500–2000 rpm. The emulsion was inverted by the addition of 150 mL of a 0.05 M CaCl2 aqueous solution. The obtained particles were recovered by filtration, washed with ethanol or acetone and subsequently with deionized water. The overall process temperature was maintained at 25°C and after preparation, the particles were stored at 4°C.

All experiments were performed in a stirred jacketed round bottom glass tank with internal diameter of 5 cm and height equal to 13 cm, employing a mechanical stirrer (model Q-251D2, Quimis) with a three tilted-blade propeller (diameter of 4 cm).

When appropriate, the statistic strategy of factorial experimental design [21] was used throughout the study. Analysis of variance (ANOVA) of the obtained data was performed through the software Statistica version 5.5 (StatSoft Co.). A significance level of *p* lower than 0.05 for particle mean
diameters was assumed to be statistically significant in this study.

2.4. Particle characterisation

The particles were assayed for morphology, mean diameter, size distribution, *A. hydrophila* incorporation efficiency and particle stability in water and in gastrointestinal conditions as follows.

The morphology of the microparticles was evaluated by optical microscopy (model DMLM, Leica), while microparticles mean volume diameters and size distribution were determined by laser scattering spectrometry (Mastersizer S, model S-MAM 5005, Malvern Instruments).

The antigenic agent incorporation efficiency was calculated as the ratio between the mass of encapsulated agent in the recovered particles and the total mass of bacteria added during particle production [11]. After dissolving 1 g of wet particles with 9 mL of 55 mM sodium citrate aqueous solution overnight [12], the samples were centrifuged at 10,000 rpm for 10 min and the resulting cell pellet was resuspended with 0.9% saline. Sample absorbance was determined at 500 nm and compared to a previously prepared standard curve.

Particle stability in aqueous solutions simulating fish gastrointestinal conditions was evaluated for samples (0.2 g of wet particles) incubated for 12 h at room temperature in 1.8 mL of deionized water at pH 2.0 (adjusted with HCl) and 4.5, PBS at pH 7.4 and Tris–buffer at pH 9.0. After incubation in the different conditions, the amount of bacteria released was determined at 500 nm in the supernatant of samples centrifuged at 500 rpm for 10 min to remove the microparticles.

3. Results and discussion

With the purpose of producing particles with adequate characteristics for fish oral immunisation, the effects of various operational and formulation factors were investigated, whose results are discussed as follows.

3.1. Effects of emulsion preparation conditions on particle characteristics

Exploratory experiments were initially designed to evaluate the influence of alginate concentrations ranging from 2 to 5% and stirring rates from 500 to 2000 rpm, at an aqueous to oil phase ratio of 1:1 (v/v) without addition of Span 80, according to a $2^3$ experimental factorial design with a central point at 3.5% alginate and 1250 rpm. The obtained particles were mostly deformed in shape, possibly because the gelification solution (15.5 mL) was added to the emulsion, resulting in, initially, only local Ca$^{2+}$ saturation (around the droplets). Intermediate to high quantities of corn oil were retained in all preparations. As shown in Table 1, the particles presented mean diameters varying from around 109 to 487 µm. When 5%
alginate was employed, the aqueous solution was too viscous and manipulation was considerably more difficult than in the other conditions. With 2% alginate and 500 rpm, the obtained emulsion was not stable and no particles were formed, therefore preventing the performance of data statistical analysis. The most uniform and spherical particles were observed in the condition corresponding to the central point, in which also the smaller particles were obtained.

As also observed by other groups, the size of the formed particles is directly associated to the viscosity and concentration of the alginate solution employed [11,15,17,22]. Low alginate concentrations favour particle size reduction as observed by Lemoine et al. when evaluating alginate concentrations in the range from 1.0 to 5.0% [12]. Nevertheless, decreased alginate concentrations can also reduce particle mechanical resistance and stability in hostile environments such as the gastrointestinal tract. Therefore, when evaluating the influence of the proportion between the volumes of alginate aqueous phase and corn oil on particle characteristics, the central point experimental conditions were employed. In the remaining part of the study, the emulsion was added to the gelification solution, aiming to more rapidly and efficiently stabilise the structure of the particles.

The results show that increasing the proportion from 10 to 50% of the aqueous phase keeping the same mixing conditions lead to increases on particle mean diameters from 56.2 ± 0.0 to 109.4 ± 9.9, apparently stabilizing after 40%, possibly as a result of the larger size of the aqueous phase droplets dispersed in the hydrophobic phase. Also, aqueous droplets coalescence was enhanced at lower corn oil volumes, since this phenomenon can only be neglected in diluted emulsions, in which the oil phase is in excess and, therefore, particle–particle contact is minimized. Oil retention was considerable in most of the recovered particles, being more intense when larger aqueous phase volumes were used.

Since the lowest particle mean diameter was achieved when the proportion of aqueous alginate to corn oil was 10%, this condition was adopted in the following studies, when the influence of Span 80 concentration in the corn oil phase and the stirring rate during the emulsion formation step was assessed through a $2^2$ experimental factorial design. The central point condition was performed in triplicate to estimate the experimental error.

According to Poncelet et al. [15], the use of Span 80 induces significant particle size reduction due to the decrease in the alginate droplet surface tension, which prevents droplet coalescence. The results in Table 2 corroborate this observation and the statistic analysis of these data indicates that increases in all tested variables effectively resulted in mean particle diameter reduction. Both independent variables and their interaction factor were statistically significant at 95% of confidence, however, the variable that most affected the system was the stirring rate. The analysis of variance showed that the correlation coefficient was 0.90 and the ratio between the calculated and the listed $F$-values was slightly above one.

The response surface shown in Fig. 2 obtained using the resulting mathematical model (despite not being considered as a good predictive model) clearly indicates that to obtain particles with low diameters, high stirring rates should be employed at any surfactant concentration in the tested range.

However, the particles prepared with 2% Span 80 were moderately deformed in comparison to the microparticles prepared employing the lower surfactant concentration, as shown in Fig. 3. Based on system behaviour indicated in Fig. 2 and on the fact that particles prepared with 0.2% surfactant at 2000 rpm were highly spherical in shape, associated to the lower
costs resulting from using less Span 80, these experimental conditions were employed in all following studies.

The substitution of corn oil by soybean, canola or sunflower oil in systems containing 0.2% Span 80 did not result in particle size reduction. With the use of soybean and canola oils, particle mean diameters increased around 8 \( \mu \text{m} \), while with sunflower oil, mean particle diameters reached 71 \( \mu \text{m} \). From the point of view of particle manufacture cost reduction, the use of soybean or corn oil is preferable.

### 3.2. A. hydrophila incorporation

To assess the ability of the alginate microparticles to incorporate the inactive bacteria, the effects of the initial \( A. \) hydrophila content on cell encapsulation efficiency were evaluated. For all initial bacteria concentrations tested (from 2.37 to 14.22 mg of bacteria per millilitre of alginate aqueous solution), around 100% of the added cells were effectively incorporated in the gel structure. These particles presented mean diameters varying from 34.5 \( \pm \) 0.7 to 80.2 \( \pm \) 3.2 \( \mu \text{m} \). When comparing particles surface morphology depicted in Fig. 4, a clear difference is noticed between rough bacterium-loaded microspheres and smooth alginate-soley containing particles.

High incorporation efficiencies are in fact expected for large molecular weight active agents [12], and the results obtained for \( A. \) hydrophila can be directly attributed to the particle production methodology. The small alginate droplets in the emulsion of bacteria-containing alginate with the corn oil added with Span 80 instantaneously gelled when in contact with the calcium ions of the gelification solution, effectively entrapping the bacteria into the ionically cross-linked alginate lattice. Increasing the initial cell concentration in the aqueous alginate solution, therefore, resulted in increased amount of cells in the gel lattice, which, in turn, augmented cell loading and, consequently, particle size.

### 3.3. Particle stability in fish gastrointestinal conditions

To obtain the appropriate immune response, the alginate particles containing the encapsulated antigen must be stable in fish gastric conditions and release the bacteria to contact the enterocytes in fish hindgut, which show antigen-transporting capacity [8]. The stability experiments performed focused the gastrointestinal conditions of Nile tilapia, one of the most popular fish consumed, and consequently, one of the major groups of farm raised fish in the world.

In the stomach of Nile tilapia, the oxytotic cells produce pepsinogen and hydrochloric acid, and shortly after feeding, the
pH can reach 2.0 and return to 4.5, remaining in this condition until the next feeding. In the posterior part of tilapia intestine, on the other hand, the pH can reach values up to 9.0. The average time of food traffic in the tilapia gastrointestinal system is around 12 h; however, water temperature, as well as food composition, granulometry and ingested amount can influence the digestion process. Therefore, to simulate tilapia’s gastric and intestinal conditions, the stability of particles containing the digestion process. Therefore, to simulate tilapia’s gastric and intestinal conditions, the stability of particles containing 14.2 mg/mL of A. hydrophila was tested in aqueous solutions at 25 °C for 12 h, in the pH range varying from 2.0 to 9.0.

The samples incubated in acidic conditions did not show significant morphology variation, and bacteria release was inferior to 6%. Therefore, the produced alginate microparticles were resistant to degradation in low pH, as also reported by other groups [8,10,23]. Bacterium release was more intense at higher pH values, but reaching only around 23% at pH 9.0 with Tris–buffer. These results were expected, since in basic conditions alginate microspheres can swell and release the encapsulated active agent [8]. Possibly, in this work, bacterium release was not too intense due to the large size of the entrapped microorganism when compared to the alginate matrix pore diameter, what would prevented its premature loss and allow efficient absorption in vivo.

4. Conclusions

In this study, an efficacious method which can be industrially implanted with low initial and production costs was described for the production of small biocompatible alginate microspheres stably entrapping inactive A. hydrophila cells for fish oral immunisation. Spherical and non aggregated alginate particles with diameters smaller than 50 μm were obtained through a controlled emulsification method employing corn oil and Span 80, resulting in bacterium incorporation efficiency and loading of around 100% and up to 14 mg/mL of wet particles, respectively. These particles were stable in gastrointestinal conditions for up to 12 h and show high technological potential as a delivery system for animal immunisation with bacteria.

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References


