Iranian Journal of Pharmaceutical Sciences
Spring 2007: 3(2): 61-68
www.ijps.ir

Original Article

Alginate Microsphere as a Delivery System and Adjuvant for Autoclaved *Leishmania major* and Quillaja Saponin: Preparation and Characterization

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Abstract

The goal of this study was to prepare and characterize alginate microspheres as an antigen delivery system and adjuvant for immunization against leishmaniasis. Microspheres encapsulated with autoclaved *Leishmania major* (ALM) and Quillaja saponin (QS) were prepared by an emulsification technique and characterized for size, encapsulation efficiency and release profile of encapsulates. Selection of appropriate parameters (viscosity of alginate, emulsifier and sonication times) enabled the preparation of alginate microspheres with a mean diameter of 1.92±1.0 μm, as determined by scanning electron microscopy and particle size analyzer. The encapsulation efficiency was about 34.2±6.7% for ALM and 31.0±4.4% for QS, as determined by spectrophotometric assays. *In vitro* release profile showed a slow release rate for encapsulated ALM and QS, 35.7±8.7% of ALM and 36.9±4.7% of QS were released during 1 week. The molecular weight was evaluated by SDS-PAGE and showed that the process of encapsulation did not affect the molecular weight of the entrapped antigen. With regard to the optimum diameter (less than 5 m), slow release rate and preservation of antigen molecules, formulated alginate microspheres could be considered as a promising antigen delivery system and adjuvant for ALM and QS.

Keywords: Adjuvant; Alginate microsphere; Immunization; *Leishmania major*; Leishmaniasis; Quillaja saponins.
Received: December 21, 2006; *Accepted:* March 15, 2007

1. Introduction

Infection with *Leishmania major*, an obligate intracellular parasite, causes cutaneous leishmaniasis in man, which affects millions of people in the world [1-3]. Control of leishmaniasis, through provision of chemotherapy and the control of animal reservoir and vector by a variety of methods, is impractical and difficult to achieve. The fact that recovery from the cutaneous lesions that normally occurs is accompanied by cell-mediated immune responses suggests that such natural protective immune mechanisms may be exploited by appropriate vaccine

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strategies designed to induce protective cell-mediated immunity [3]. As such, recently several immunoadjuvant (e.g. BCG and G-CSF) have been tested to potentiate the immune responses [4, 5].

Sodium alginate is a naturally occurring polysaccharide which can be easily cross-linked into a solid matrix with the addition of di- or trivalent cations (cross-linking in a water-in-oil emulsion results in the formation of microspheres). Alginate microparticles are safe to be used in animals and they have been used to encapsulate proteins. Recently, alginate microspheres have been used in several immunization studies [6-8]. Immunization of animals with alginate microspheres containing antigenic proteins elicited both humoral and cell-mediated immune responses [9].

The immunoadjuvant effect of Quillaja saponins (QS), extracted from *Quillaja saponaria* bark, has been shown in several studies [10-15]. Saponins could induce Th1 cells, CD_8^+ cytotoxic T lymphocytes (CTL) and, IgG2a and IgG2b antibody responses [15]. The possible mechanism of immunoadjuvant effects of saponin is formation of mixed-micelle with cell proteins [14, 15]. Controlling the cytokine production, clonal differentiation of complement cells in lymph nodes, enhancing the CTL and natural killer cell (NKC) activity, mitotic effect on lymphocytes and activation of macrophages and granulocytes have also been attributed to saponins [13-16].

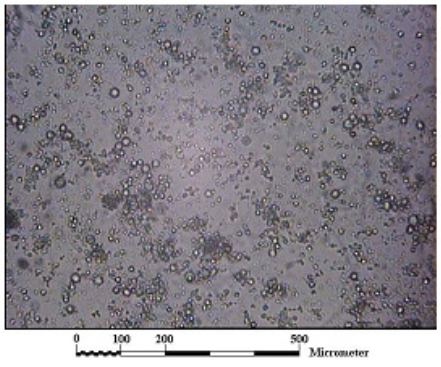

Figure 1. Optical microscope pictures from alginate microspheres.

The immunogenicity of the antigen and potency of the adjuvant was substantially enhanced by co-delivery in biodegradable microspheres [17]. Therefore, the aim of this study was to prepare and characterize alginate microspheres encapsulated with autoclaved *Leishmania major* (ALM) and QS adjuvant for immunization against leishmaniasis.

2. Materials and methods

2.1. Materials

Sodium alginate (low viscosity grade) and purified QS were purchased from Sigma (St Louis, MO, USA). The surfactants (Span-60 and Span-85) and bovine serum albumin (BSA) were obtained from Fluka (Buchs, Switzerland). ALM was provided by Razi Inc. (Hesarak, Karaj, Iran). Calcium chloride, n-octanol, sodium citrate and isopropyl alcohol were from Merck (Darmstadt, Germany). All chemicals were of analytical grade and were used as received.

2.2 Methods

2.2.1. Preparation of alginate microspheres encapsulated with ALM and QS

An emulsification method was used for preparation of alginate microspheres [18, 19]. Briefly, an aqueous solution containing sodium alginate (3.0% w/v, low viscosity) was dispersed in a n-octanol solution containing a lipophilic surfactant (2.0% w/v, Span-85) by using a mechanical homogenizer (Ultra-turrax T8, Germany) at 21000 rpm or by probe sonication (Soniprep150, MSE, Sussex, UK). In the case of ALM and QS loaded microspheres, 3 mg of ALM and 20 µl QS (10 mg/ml in distilled water) were added in the aqueous solution containing sodium alginate. The W/O emulsion was rapidly added to a solution of calcium chloride in octanol (60 ml, 0.33% w/v) while stirring the whole medium slowly with a magnetic stirrer.

After 10 min. 2 ml isopropyl alcohol was added dropwise to harden the formed microspheres. The microspheres were collected by filtration, washed with isopropyl alcohol and finally dried in a vacuum desiccator.

2.2.2. Particle size determination

Optical microscope (Olympus, Germany) was used for studying the morphology and size distribution of microspheres. For the latter purpose the diameter of 300 microspheres was determined under the optical microscope equipped with an eyepiece reticule. Particle size (volume mean diameter) and size distribution of the microspheres was determined using a particle size analyzer (Malvern, UK).

2.2.3. Encapsulation efficiency of ALM and CpG-ODN in alginate microspheres

For determination of the loading of ALM in alginate microspheres, known amounts of ALM loaded microspheres were accurately weighed and completely dissolved in sodium citrate solution (0.1 M, pH 7.4) [19]. The Lowry protein assay method [20] was used to determine the ALM concentration in the solution.

Microspheres containing only QS were similarly dissolved in sodium citrate solution and the amount of QS was estimated spectrophotometrically by Lowry protein assay method. As both ALM and QS have absorbance in Lowry method, it was not possible to quantify QS and ALM simultaneously. Therefore, QS formulated in microspheres under similar conditions without the protein, was used to estimate the encapsulation efficiency. For each batch of microspheres the encapsulation efficiency was determined in triplicates.

2.2.4. In vitro release studies of ALM and QS

Alginate microspheres (30 mg) containing ALM were suspended in 600 µl phosphate buffered saline (PBS, 10 mM, pH 7.4, containing 0.01% sodium azide). The suspensions were then incubated at 37 °C under continuous shaking for 1 weak. At various time intervals (0.5, 1, 2, 4, 12, 24, 48 and 168 h), the supernatant (500 µl) was drawn after centrifugation (5000g for 5 min.)

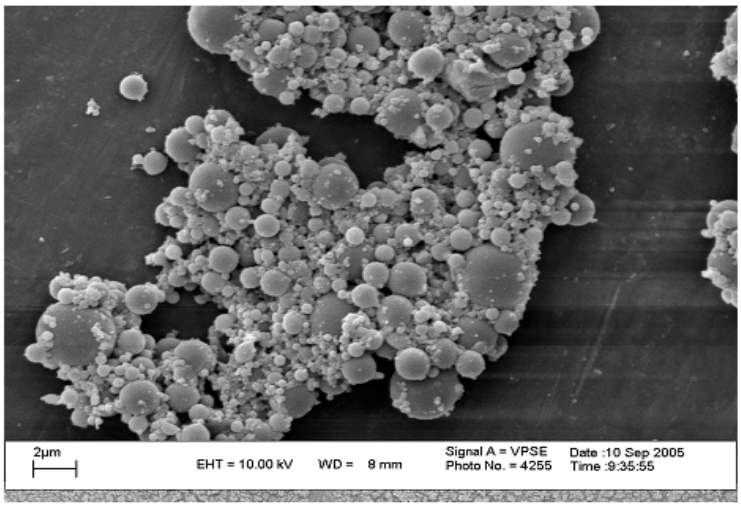

Figure 2. Scanning electron micrograph of alginate microspheres.

and replaced with fresh medium [17, 19]. ALM released into the supernatant was quantified by Lowry protein assay method.

Similarly, QS containing microspheres were incubated in the same condition and the released fraction was estimated spectrophotometrically by Lowry protein assay method. Each *in vitro* release study was performed in triplicates.

2.2.5. Structural stability (molecular weight) of encapsulated antigen

The molecular weight and integrity of encapsulated ALM was determined by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE). The ALM released from dissolved microspheres, original ALM and a molecular weight reference marker (molecular weight 14-66 kDa) were loaded onto 10% acrylamide gel and run using electrophoresis system (paya-pajoohesh, Iran). Protein bands were visualized by silver nitrate staining [20].

2.2.6. Statistical analysis

Statistical analysis of the results was carried out using unpaired t-test.

3. Results and discussion

3.1. Size determination of alginate microspheres

It has been shown that particulate antigens are better sampled with antigen presenting cells (APCs) than the soluble antigens [21]. Particle size has a determinative role in interaction with APCs. Particles with diameters smaller than 10 µm can be directly taken up by macrophages and dendritic cells through phagocytosis [22], whereas larger microspheres (greater than 10 µm) need to undergo biodegradation, before phagocytosis can occur. Degradation, antigen release, location and antigen presentation of microspheres smaller than 10 µm are expected to be different from larger ones [23].

Induction of immune responses following enteric immunization with porcine serum albumin encapsulated in alginate microspheres was studied by Mutwiri *et al.* The volume diameter of microspheres was 9-14 µm and greater than 90% of particles had a diameter less than 10 µm. The microspheres could induce the humoral immune responses, but failed to activate the cellular immunity [24].

Alginate microspheres were used for encapsulation of DNA, adenovirus or both and to study the effect of route of inoculation (sc, im, ip, oral and nasal) on immune response. The majority of microspheres were of 5-10 µm in diameter [9]. In another study rotavirus antigens encapsulated in alginate microspheres were used in oral immunization. Microspheres were prepared by spray method and 80% of the microspheres had a diameter of 10 µm or less. The highest level of virus-specific fecal IgA antibody, similar to responses induced by oral immunization with live PRV, was induced [25].

For immunization with polysaccharide-protein conjugates entrapped in alginate microspheres, Cho *et al.* prepared microspheres that had diameters of less than 5 µm. Microspheres evoked both the mucosal IgA and systemic IgM responses [26]. For induction of pulmonary immunity in cattle, alginate microspheres encapsulated with ovalbumin were orally administered. The distribution of size of alginate microspheres

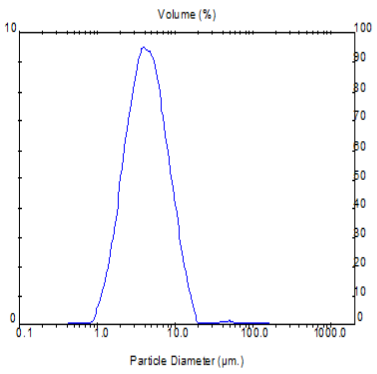

Figure 3. Size distribution curve of alginate microspheres. Particle size (volume mean diameter) and size distribution of the microspheres was determined using a particle size analyzer.

ranged from <1 μm to >50 μm in diameter, with 70% of the microspheres less than 10 μm in diameter [27].

In this study by considering the optimum formulation parameters from our previous studies [19, 28], microspheres with number mean diameter of 1.92±1.0 μm were prepared. Percentage of microspheres with diameters larger than 10 μm, was less than 10%. In our previous studies and also other studies concluded that parameters such as alginate molecular weight and concentration, surfactant type and concentration, sonication time and concentration of Ca^{2+} could affect the particle size and morphology. Among these parameters, the surfactant type (Span 85 with less HLB than Span 60, showed better results) and alginate MW and concentration showed to be more effective on microspheres diameters [7, 19, 22, 28].

3.2. Loading of ALM and QS in alginate microspheres

A protein delivery system with high loading capacity is very advantageous, because it can prevent the loss of antigen and also limit the need of administering high level of carrier [18]. These are several reports on the encapsulation of proteins in alginate microspheres [6, 7, 18, 25-27, 29]. Encapsulation efficiency of both rotavirus protein and virus in alginate microspheres was approximately 30% [25]. The loading of ovalbumin in alginate microspheres showed a similar results of 33.33% [27], while 60% loading was gained for polysaccharide-protein

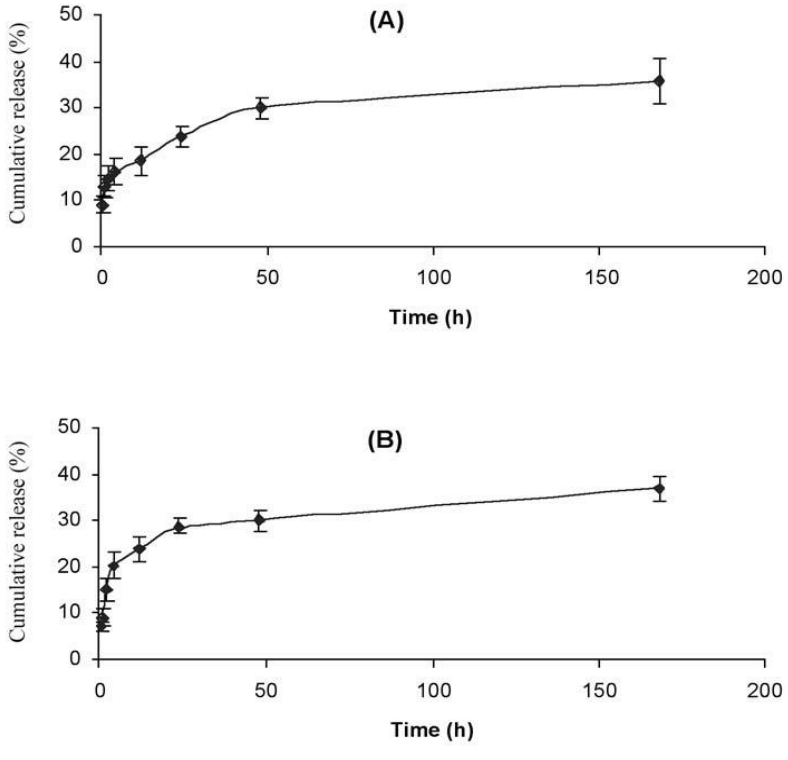

Figure 4. *In vitro* release of encapsulated ALM (A) and Quillaja Saponin (B) from alginate microspheres. Microspheres were suspended in PBS buffer at 37 °C under continuous shaking. At various times the cumulative amounts of released ALM (A) and QS (B) were quantified. Error bars represents the S.E. (n=3).

conjugates [26]. In our previous studies the loading of tetanus toxoid and CpG-ODN was determined as 47.7% and 34.2% [18].

Differences amongst data come from the variations in morphology, porosity and the size of microspheres prepared, the molecular weight of encapsulated protein, the viscosity of sodium alginate solution, the nature of surfactant used to stabilize W/O emulsion and some other factors [19, 30, 31].

At the present study, loading of the ALM and QS was respectively determined as 34.2±6.7% and 31±4.2%. The loadings are similar to the majority of other experiences with alginate microspheres [18, 25, 27].

3.4. In vitro release profiles of ALM and QS from alginate microspheres

The polymeric microspheres are generally utilized either for prolonging the circulation half-lives of proteins or for targeted delivery to specific tissues. Alginate matrices have been proven to be useful for the slow release of several potential therapeutic proteins and several studies have demonstrated the usefulness of these systems [18, 29]. The release profiles of ALM and QS from microspheres were evaluated in PBS. ALM was released from microspheres with a burst release of 9.1±3.0% after 30 min. This was followed by a slow and continuous manner. After 7 days, 35.7±8.7% of antigen was released (Figure 4A). The release of QS showed a burst effect of 7.3±1.9% after 30 min. After 7 days of incubation, cumulative release of 36.9±4.7% was observed (Figure 4B).

In other studies, the release of polysaccharide-protein (PS) conjugates from alginate microspheres was significantly higher than the present study [26]; 80% of PS released within one day, while the released ALM in the same time was 24%. In the release of tetanus toxoid from alginate microspheres, prepared with the same method, low release rate (21.6% of TT release after 1 week) was observed [19]. In another study in our lab, the release of ALM from PLGA nanospheres (mean diameter of 300±123 nm) during the first week of study reached to 44.8% (unpublished data). These results indicated that alginate microspheres prepared with this method could effectively control the release profile of encapsulated antigen. It has been shown that particulate antigens, compared with soluble antigens, are better phagocyted by antigen presenting cells and so, they have more potentials for induction of immune responses [17]. Alginate microspheres could be used as a delivery system and adjuvant to give to the ALM a particulate entity. Thus, the less antigen release rate is better, because more antigens keep their particulate nature [18].

3.5. Structural stability of encapsulated antigen

A critical point in developing a carrier system for antigens is the preservation of their native molecular weight. During alginate microsphere preparation, ALM 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 antigenicity

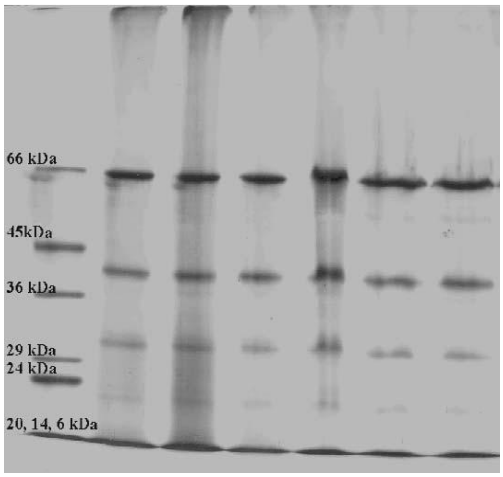

Figure 5. Polyacrylamide gel electrophoresis of encapsulated ALM. From left, the first band is molecular weight maker. The latter three bands are native ALM and the last three bands are encapsulated ALM.

of protein. Therefore molecular weight of ALM encapsulated in microspheres was evaluated by SDS-PAGE. In the SDS-PAGE gel, identical bands were observed for the native ALM and the ALM released from alginate microsphere. Hence the data suggest that the molecular weight of ALM was not affected by the entrapment procedure (Figure 5). Previous studies also showed similar results [7, 18, 32].

4. Conclusion

The present study demonstrated that alginate microspheres with optimum characteristics can be prepared by emulsification method. Microspheres had a mean diameter of 1.92±1.00 μm. More than 90% of microspheres were under 10 μm in diameter. An acceptable encapsulation efficiency for ALM (34.2±6.7%) and QS (31±4.2%) were achieved. The release profiles of encapsulated ALM and QS showed that this delivery system can be used successfully to keep the particular properties of antigen for several days. The structural integrity of encapsulated ALM was not affected by encapsulation procedure. All these characteristics make the alginate microspheres encapsulated with ALM and QS adjuvant, particularly interesting for immunization against leishmaniasis.

Acknowledgements

This study was part of a research project which was financially supported by the vice chancellor for research of Mashhad University of Medical Sciences. Also Dr. Gilani from the Department of Pharmaceutics, Faculty of Pharmacy, Tehran University of Medical Sciences is greatly acknowledged for his cooperation in particle size analysis.

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