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Preparation and *In Vitro* Characterization of Alginate Microspheres Encapsulated with Autoclaved *Leishmania major* (ALM) and CpG -ODN

[1]Z. Ghiasi, [2] S. A. Sajadi Tabasi, *[3]M. Tafaghodi

Abstract

Objective

The goal of this study was to prepare and characterize alginate microspheres as an antigen delivery system and adjuvant for immunization against leishmaniasis.

Materials and Methods

Microspheres 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 1.8 ± 1.0μm, as determined by Scanning Electron Microscopy and Particle Size Analyzer.

Results

The encapsulation efficiency was about 34.2 ± 6.7% for autoclaved leishmania major and 63.5 ± 6.9% for CpG-ODN, as determined by spectrophotometric assays. *In vitro* release profile showed a slow release rate for encapsulated ALM, while higher release rate was observed for CpG-ODN. 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.

Conclusion

With regard to the optimum diameter (less than 5 μm), slow release rate and preservation of antigen molecules, alginate microspheres could be considered as a promising antigen delivery system for ALM.

Keywords: Adjuvant, Alginate microsphere, CpG-ODN, Immunization, Leishmaniasis.

1- School of Pharmacy, Mashhad University of Medical Sciences, P.O. Box 91775-1365, Mashhad, Iran
2 - School of Pharmacy and Pharmacological Research Center of Medicinal Plants, Mashhad University of Medical Sciences, Mashhad, Iran
3 - School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran
*Corresponding author: Tel: +98- 511- 8823255; Fax:+98- 511- 8823251; email: tafaghodim@mums.ac.ir

Introduction

Leishmaniasis, caused by obligate intracellular parasites of the genus *Leishmania*, affects millions of people in the tropical and subtropical regions of the world, causing a spectrum of manifestations ranging from asymptomatic infection and mild self-healing cutaneous disease to severe non-healing diffuse cutaneous and visceral leishmaniasis (1,2). 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. As such, vaccination becomes an ideal effective method of control (1,3) and recently, several immunoadjuvants (e.g. BCG and G-CSF) have been tested to potentiate the immune responses (3,4).

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 (5-7). Immunization of animals with alginate microspheres containing antigenic proteins elicited both humoral and cell-mediated immune responses (8).

Oligodeoxynucleotides (ODN) containing unmethylated CpG motifs act as immune adjuvants, boosting the humoral and cellular responses to co-administered antigens (9,10). There are data demonstrating that CpG-ODN, when used as a vaccine adjuvant with leishmania antigen, can induce long-term protection against an intracellular infection in a CD +8 and CD +4 dependants (11-13).The immunogenicity of the antigen and potency of the adjuvant was substantially enhanced by co-delivery in biodegradable microspheres (14). Therefore, the aim of this study was to prepare and characterize alginate microspheres encapsulated with autoclaved leishmania major (ALM) and CpG-ODN adjuvant for immunization against leishmaniasis.

Materials and Methods

Materials

Sodium alginate (low and medium viscosity grade) was 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). Autoclaved leishmania major (ALM) was provided by Razi Inc. (Hesarak, Karaj, Iran). CpG oligodeoxynucleotide (# 1826, seq (5′-3′): tccatgacgttcctgacgtt) was purchased from Microsynth (Switzerland). 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.

Methods

Preparation of alginate microspheres encapsulated with ALM and CpG-ODN

An emulsification method was used for preparation of alginate microspheres (15-17). Briefly, an aqueous solution containing sodium alginate (3.0% w/v, low or medium viscosity) was dispersed in a n-octanol solution containing a lipophilic surfactant (2.0% w/v, Span-85 or Span-60) 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 CpG-ODN loaded microspheres, 3 mg of ALM and 50 µl of CpG-ODN (5 mg/ml in TE buffer) 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.

In order to prepare alginate microspheres having a diameter less than 10 µm and high load, the effect of surfactant nature and the alginate molecular weight and the influence of sonication time were studied. Six batches of microspheres were prepared and marked as B-1 to B-6 (Table 1). For each variable studied, batches of microspheres were prepared in triplicate.

Table 1. Various parameters investigated to optimize microspheres preparation.

Batch	Surfactant	Alginate viscosity	Emulsification
B-1	Span 60	Medium	Homogenizer
B-2	Span 85	Medium	Homogenizer
B-3	Span 85	Low	Homogenizer
B-4	Span 85	Low	Sonication 3×30s
B-5	Span 85	Low	Sonication 2×30s
B-6	Span 85	Low	Sonication 1×30s

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 of the batch-6 was determined using a particle size analyzer (Malvern, UK).

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) (17). The Lowry protein assay method (18) was used to determine the ALM concentration in the solution.

Microspheres containing only CpG-ODN were similarly dissolved in sodium citrate solution and the amount of ODN was estimated spectrophotometrically based on absorbance at 260 nm (19). As ALM itself shows absorbance at 260 nm, it was not possible to quantify ODN in its presence. Therefore, CpG-ODN 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.

In vitro release studies of ALM and CpG-ODN

Alginate microspheres (30 mg) containing ALM were suspended in 600 µl of phosphate buffered saline (PBS, 10 mM, pH 7.4, containing 0.01% sodium azide). The suspensions were then incubated at 37 ºC under continuous shaking for 1 week. At various time intervals (0.5, 1, 2, 4, 12, 24, 48 and 168 h), the supernatant (500 µl) was removed after centrifugation (5000 g for 5 min) and replaced with fresh medium (14,17). ALM released into the supernatant as quantified by Lowry protein assay method. Similarly, CpG-ODN containing microspheres were incubated in the same condition and the released fraction was estimated spectrophotometrically based on absorbance at 260 nm (19). Each *in vitro* release study was performed in triplicates.

Structural stability (molecular weight) of encapsulated antigen

The molecular weight 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 (18).

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

Results and Discussion

Size determination of alginate microspheres
Microsphere size is an important parameter. Small particles, with sizes smaller than 10 µm can be directly taken up by macrophages and dendritic cells through phagocytosis. It is an important property for stimulating the immune system, 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 (20). So, in order to prepare small alginate microspheres (<10 µm), the effect of formulation variables (surfactant nature, alginate molecular weight and sonication) on alginate microsphere size were investigated. The influence of each variable on size features (mean diameter, size range and percentage of microspheres with diameter greater than 10µm) was compared using optical microscope (Table 2, Figure. 1A-1D).

Table 2. Size determination of alginate microspheres(optical microscope).

Batch	Mean diameter (µm)	Size range (µm)	Percent over 10 µm	Microsphere yield %
B-1	Sever clumping , without particulate property			
B-2	15.7 ± 5.9	5 - 80	53.3 ± 6.6 %	64 ± 9
B-3	9.2 ± 5.0	2.5 - 70	33.4 ± 6.5 %	-
B-4	3.3 ± 1.5	1 - 22.5	2 ± 0.2 %	86 ± 5
B-5	3.1 ± 2.3	1 - 20	2.3 ± 0.4 %	133 ± 45
B-6	6.5 ± 3.1	1 - 35	25.3 ± 0.6 %	106 ± 13

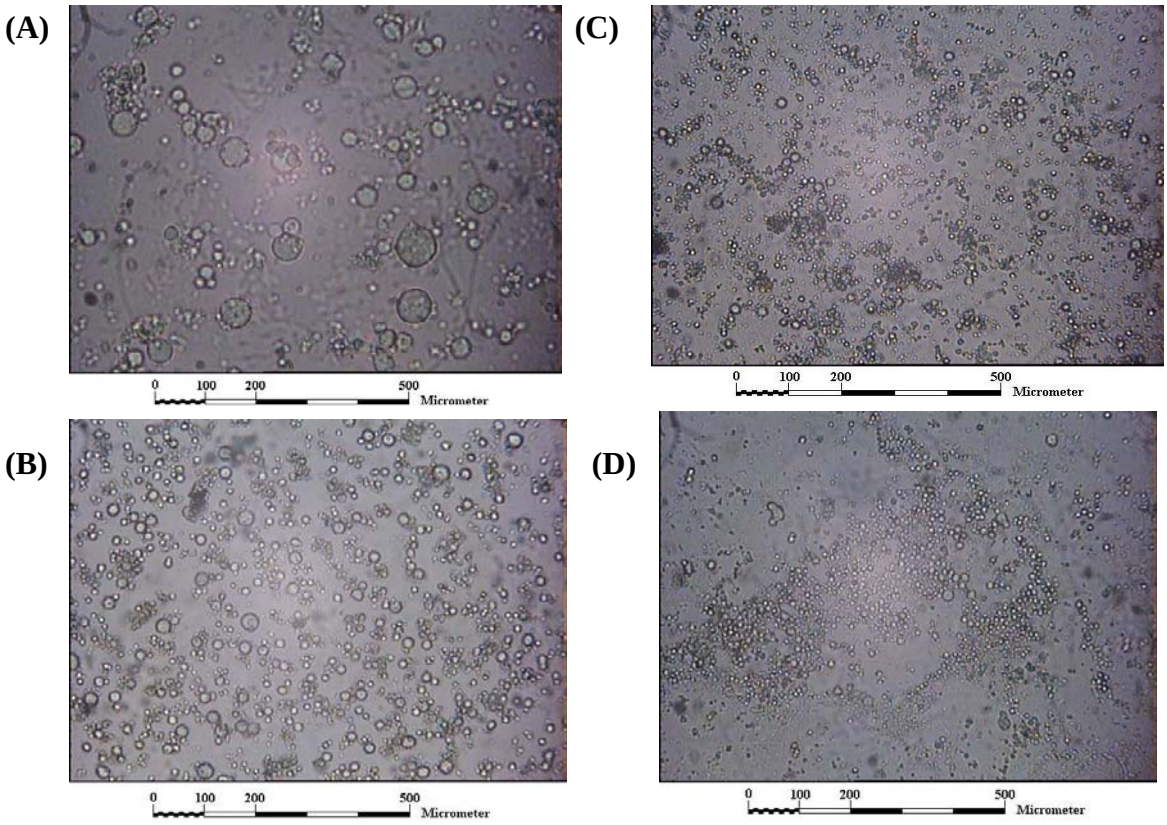

Figure 1. Optical microscope pictures from alginate microspheres of batches B-2 (A), B-4 (B), B-5 (C) and B-6 (D).

Since the present method of microsphere preparation involves emulisification, the surfactant could play an important role in the microsphere production (21). The emulsion required is an water-in-oil (w/o) system, so surfactants with lower HLB, will be optimal. This study showed that Span 60 can not give proper microspheres. This could be due to its HLB which is not as low as required (4,7), as well as to the nature of the surfactant that couldn't produce a fine emulsion, whereas Span 85 (HLB = 1.8) could produce smaller aqueous droplets during the emulsification (Batches B-1 and B-2, Table 2). It has also been used by other researchers (6).

The influence of alginate molecular weight was evaluated. Low viscosity grade alginate provided smaller microspheres (Batch B-3, 9.2 ± 5.0µm) as compared to medium viscosity grade alginate (Batch B-2, 15.7 ± 5.9µm) ($p<0.05$) (Table 2, Figure. 1A-1D). It has been shown that by increasing the molecular weight of alginate, which would result in higher viscosity of solution, the microsphere size would also increase (6,17). Therefore, sodium alginate with low viscosity grade was used in the further preparations.

Since the emulsified alginate droplets form microspheres, the emulsification procedure and resulting emulsion have important effects on morphological features of final microspheres. Sonication of microspheres prepared with low viscosity grade alginate, significantly reduced the mean diameter of microspheres, size range, and percentage of microspheres over 10 µm in diameter (Batches B-4, B-5 and B-6 compared with Batch B-3, $P<0.05$, Table 2). This could be due to the ability of ultrasonic waves to prepare the micron sized droplets (17).

Increasing the sonication time results in decrease in size of aqueous droplets. By increasing the sonication time, some very small microdrops are aggregated together (17). This could explain why the mean diameter of B-6 batch is higher than batches B-5 and B-4 (Batch B-6 compared with batches B-5 and B-6, $p<0.05$, Table 2).

As the primary studies revealed that the B-5 batch has the most appropriate morphological and size features, this batch was further studied by particle size analyzer (PSA) (Figure 2) and scanning electron microscope (SEM) (Figure 3). The volume mean diameter of microspheres was determined as 1.8 ± 1.0 µm. Less than 10% of microspheres were larger than 10 µm in diameter.

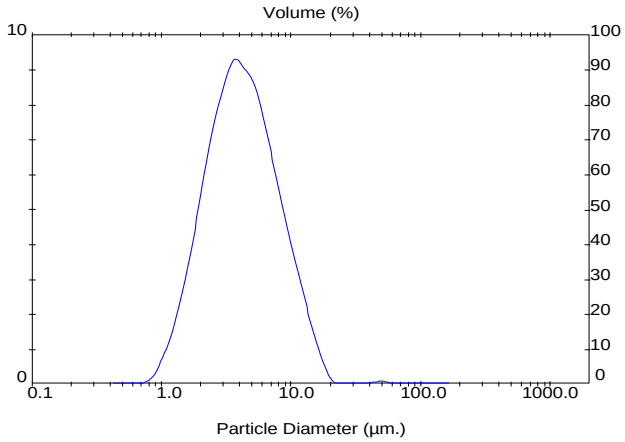

Figure 2. Size distribution curve of B-5 batch. Particle size (volume mean diameter) and size distribution of the microspheres of the batch-6 was determined using a particle size analyzer.

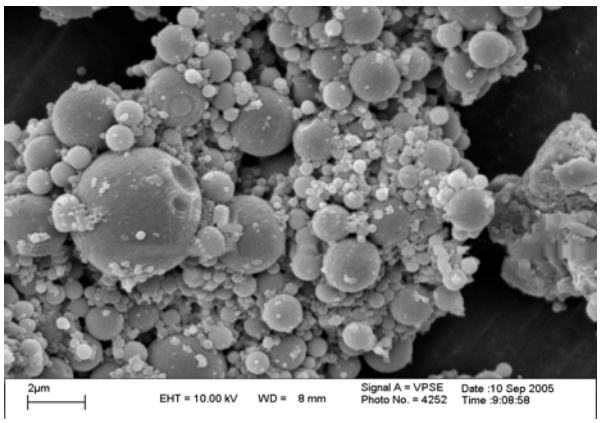

Figure 3. Scanning electron micrograph of alginate microspheres (B-5 batch).

Loading of ALM and CpG-ODN in alginate microspheres

To assess the ability of alginate microspheres to encapsulate protein, the encapsulation efficiency was studied. 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 (16).

Numerous reports have been published on the encapsulation of proteins in alginate microspheres (5, 6, 16, 22). Differences among 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 (17,23,24).

The results of encapsulation efficiency studies are summarized in Table 3. Regarding the porous nature of alginate microspheres, the encapsulation efficiencies are usually lower than the PLGA microspheres (14), but other advantages of alginate microspheres could cover this insufficiency. Increasing the sonication time lowered the encapsulation efficiency (comparing Batch B-4 and Batch B-5, $p<0.05$) due to droplet coalescence and increased probability of contact between the internal solution and the external phase resulting in protein loss (17, 23, 24).

As showed in Table 3, the encapsulation efficiency was very high for CpG-ODN although the molecular weight of CpG-ODN is lower than ALM. The high encapsulation efficiency can be explained by the ionic interactions occurring between the polycation CpG-ODN and the highly negatively charged alginate polymer (5,16).

Table 3. Encapsulation efficiency of ALM and CpG-ODN in microspheres.

Batch	ALM encapsulation efficiency %	ALM encapsulation range	CpG-ODN encapsulation efficiency %	CpG-ODN encapsulation range
B-4	14.3 ± 3.9	9.8 - 18.4	25.2 ± 5.9	21.6 - 28.8
B-5	34.2 ± 6.7	25.5 - 49.2	63.5 ± 6.9	55.8 - 70.6

In vitro release profiles of ALM and CpG-ODN 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 (16,22).

The release profiles of ALM and CpG-ODN from B-5 batch, which had the best size and encapsulation features, 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, a total of 35.7 ± 8.7% antigen release was occurred (Figure 4A).

The release of CpG-ODN, a much smaller molecule (MW 6363 Da) as compared to ALM (MW about 500 kDa), was apparently faster (14,16). The burst effect was seen as 28.3 ± 5.2% and after 7 days of incubation, 58.3 ± 4.1% cumulative release was observed (Fig 4B). In comparison of release profile of ALM and CpG-ODN from the microspheres, the lower release of ALM could be attributed to its higher molecular weight and also its particulate nature (ALM is suspension, while CpG-ODN is in solution form). The electrostatic interaction of CpG-ODN (positive charge) with the anionic matrix of alginate should decrease the release rate of CpG-ODN, compared with ALM. But possibly the charge attraction factor has been overshadowed with the molecular weight and particulate nature of ALM.

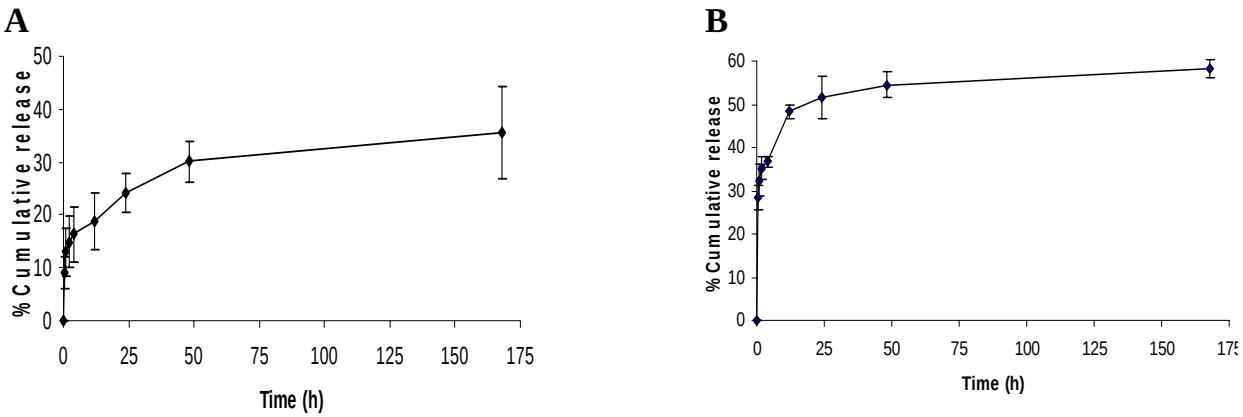

Figure 4. *In vitro* release of encapsulated ALM (A) and CpG-ODN (B) from alginate microspheres (B-5 batch). Microspheres were suspended in PBS buffer at 37 ºC under continuous shaking. At various times the cumulative amounts of released ALM (A) and CpG-ODN (B) were quantified. Error bars represents the SEM (n=3).

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 (14). 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 the better, because more antigens keep their particulate nature (16).

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 antigenicity of protein. Therefore molecular weight of ALM encapsulated in the most optimum batch of microspheres (Batch B-5) 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 suggested that the Molecular weight of ALM was not affected by the entrapment procedure (Figure 5). Previous studies also showed similar results (6,16,25).

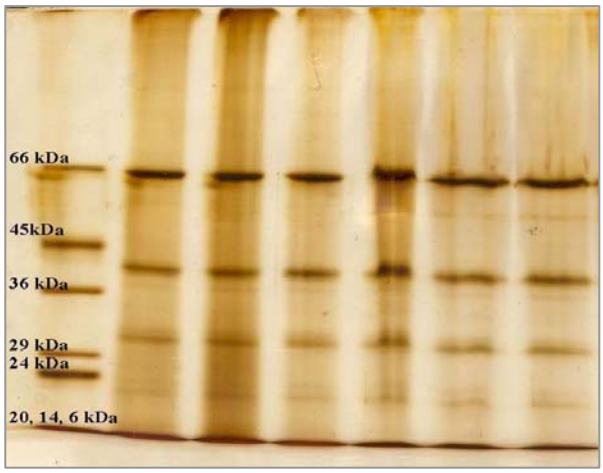

Figure 5. Polyacrylamide gel electrophoresis of encapsulated ALM. From left, respectively, the first band is molecular weight maker. The next three bands are native ALM and the last three bands are encapsulated ALM (B-5 batch).

Conclusion

The present study demonstrated that alginate microspheres with optimum characteristics can be prepared by emulsification method.

The optimum microspheres had a volume mean diameter less than 5 µm. A reasonable encapsulation efficiency for ALM (32.4 ± 6.75%) and CpG-ODN (63.5 ± 6.9%) were achieved. The release profiles of encapsulated ALM and CpG-ODN 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 autoclaved leishmania major (ALM) and CpG-ODN adjuvant, particularly interesting for immunization against leishmaniasis.

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