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***Original Article***

# *Preparation and in-vitro characterization of alginate microspheres incorporating leptospiral antigens as a delivery system and adjuvant*

***Inanlou [1,] F., Khaki [*2] P., Mohammadpour [3], N., Zolfagharian [3], H.***

*1. Department of Microbiology, Faculty Veterinary Medicine, Islamic Azad University, Karaj Branch, Karaj, Iran*
*2. Department of Microbiology, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran*
*3. Department of Venom and Human Sera, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran*

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Corresponding Author: P.khaki@rvsri.ac.ir

***ABSTRACT***

Leptospirosis is one of the most prevalent zoonotic diseases worldwide. Currently, multivalent whole-cell leptospiral vaccines can induce protection against leptospirosis. Therefore, preparation and formulation of new generations of vaccines that can stimulate long-term immunity for leptospirosis control are essential. The aim of this study was to prepare and characterize alginate microspheres as an antigen delivery system for immunization against leptospirosis. We used five Leptospira interrogans serovars, namely, *Icterohaemorrhagiae*, *Grippotyphosa*, *Serjo harjo*, *Pomona*, and *Canicola*, for antigen preparation. Alginate microspheres containing leptospiral antigen (LA) were prepared by an emulsification method and evaluated with respect to morphology, size distribution, loading efficiency (LE), loading capacity (LC), and release profile. The effects of concentration of alginate and emulsifiers and stirring rate on characteristics of microspheres were investigated. The optimal condition parameters for the preparation of LA-loaded alginate microspheres were estimated. The optimum concentrations obtained for alginate and emulsifiers were 3.65% (w/v), Span 80 (0.24% w/v), and Tween 80 (3.85% w/v), respectively. Moreover, the appropriate homogenization rate was 500 rpm. Our results showed mean particle size of 200 µm, 97.41% LE, and 8% LC for the microspheres. Sufficient release profile was observed for in-vitro release test of LA from alginate microspheres over an extended period of time (216 hour). Therefore, alginate microspheres technologically seem to be a promising antigen delivery system for leptospiral vaccine.

***Keywords:*** *Leptospira spp*, Leptospiral antigen, Alginate, Microspheres, Antigen delivery, Immunization

***Préparation et caractérisation in-vitro des microsphères d'alginate chargées d'antigènes leptospiraux en tant que système de délivrance et adjuvant***

***Résumé:*** La leptospirose est l'une des zoonoses les plus courantes à travers le monde. Actuellement, la protection contre la leptospirose peut être induite par un vaccin constitué de germes leptospiraux entiers. De ce fait, la préparation et la formulation d'une nouvelle génération de vaccins capable de favoriser une immunité à long terme sont essentielles pour le contrôle de cette maladie. L'objectif de cette étude était de préparer et caractériser un nouveau système de délivrance composé de microsphères d'alginate pour l'immunisation contre la leptospirose. Pour la préparation des antigènes, 5 sérotypes de *Leptospira interrogans* à savoir *Icterohaemorrhagiae*, *Grippotyphosa*, *Serjo harjo*, *Pomona*, et *Canicola* on été utilisés. Les microsphères d'alginate contenant les antigènes leptospiraux (AL) ont été préparées par émulsification et ensuite analysées en termes de morphologie, granulométrie, efficacité de chargement (EC), capacité de chargement (CC) et profil de libération. De plus, l'influence des concentrations en alginate et en émulsifiant a également été étudiée. Enfin, les conditions optimales de préparation pour les microsphères d'alginate chargées d'AL ont été déterminées. Selon nos résultats, les concentrations optimales de préparation étaient respectivement de 3,65% p/v (pourcentage de poids par volume) d'alginate dans l'émulsifiant, 0,24% v/v de Span 80 et 3,85% v/v de Tween 80. De plus, le taux d'homogénéisation approprié a été obtenu à 500 rpm. La taille moyenne des particules générées était de 200 nm avec un EC de 97,41% et un CC de 8%. Les

testes de libération des AL à partir des microsphères d'alginate montrent un profil de libération étendu sur une période prolongée de 216 heures. Pa conséquent, sur le plan technique, les microsphères d'alginate semblent représenter un système de délivrance prometteur pour le vaccin leptospiral.

***Mots clés:*** *Leptospira spp*, Antigène leptospiral, Alginate, Microsphères, Délivrance d'antigène, Immunisation

## *INTRODUCTION*

Leptospirosis is a serious worldwide zoonosis caused by pathogenic *Leptospira spp* and is identified as a re-emerging infectious disease, particularly in tropical and subtropical regions Yan et al. (2009). Transmission to humans occurs via contaminated water or soil by urine of infected animals. Moreover, the overall disease burden is underestimated, since leptospirosis is a significant cause of undifferentiated fever and frequently goes undiagnosed. Barriers to addressing this problem is the lack of adequate diagnostic tests and effective control measures. The currently available vaccines impart only short-term immunity, mediated by opsonizing antibodies and fail to provide cross-protection against a large number of pathogenic serovars (Andre-Fontaine et al., 2003). Although cell-mediated immunity is mostly directed against intracellular pathogens, several studies demonstrated that protective immunity against *Leptospira*, is correlated with Th1 responses and characterized by CD4+ and γδ T cell production of IFN- γ (Klimpel et al., 2003). Accordingly, an ideal vaccine against leptospirosis would activate both humoral and cell-mediated immune responses (Faisal et al., 2009a). Although a number of potent adjuvants are available, adverse reactions due to toxicity have limited their use in vaccine formulation. Aluminum hydroxide (Alum) is the only adjuvant approved for human use, which primarily induces humoral immune responses and only provides limited cell-mediated immunity. In addition, Alum is not effective for induction of mucosal immunity and can cause allergic reactions in some cases (Gupta, 1998). The particulate antigen-delivery systems (e.g., microspheres, liposomes, and virosomes) are highly potent adjuvants and are widely used against various infectious diseases (Behboudi et al., 1995). Microencapsulation is a process in which the cells are retained within an encapsulating matrix or membrane. Microencapsulation of antigens was investigated for improving their immunogenicity. This process provides a long-term depot for antigens and leads to effective and long-lasting immunity (Krasaekoopt et al., 2004). Several particulate antigens, containing live or killed microorganisms, were proved to be effective immunogens using biodegradable polymers such as poly(lactic-co-glycolic acid) (PLGA), poly(l-lactic acid) (PLLA), and alginate (Flick-Smith et al., 2002; Yeh et al., 2002; Florindo et al., 2008). Immunization studies against leptospirosis via PLGA microspheres and liposomes revealed that these particles are promising adjutants for use in vaccines against leptospirosis (Faisal et al., 2009a; Faisal et al., 2009b). The most widely applied encapsulating material is alginate, a linear heteropolysaccharide of D-mannuronic and L-guluronic acid, which is extracted from various species of algae (Krasaekoopt et al., 2004). Recently, alginate microspheres have been employed in various immunization studies (Dobakhti et al., 2006; Tafaghodi et al., 2007; Arenas-Gamboa et al., 2008). There is a paucity of data regarding the use of alginate microspheres as an adjuvant in vaccines against leptospirosis. Therefore, in the present study, we investigated the optimum conditions for preparation of alginate microspheres containing leptospiral antigens as a delivery system for immunization against leptospirosis.

## *MATERIALS AND METHODS*

***Materials.*** Low viscosity sodium alginate (Sigma Chemical Co., USA), calcium chloride, Span 80, Tween 80, and iso-octane (Merck, Germany) were employed for particle preparation. All the other applied reagents were at least of analytical grade.

***Leptospira culture and antigen preparation.*** *Leptospira interrogans* serovars Icterohaemorragia (RTCC 2812), Gripotiphosa (RTCC 2825), Serjo harjo (RTCC 2821), Pomona (RTCC 2815), and Canicola (RTCC 2824) were obtained from the Leptospira Reference Laboratory, Razi Vaccine and Serum Research Institute, Karj, Iran. The bacteria were inoculated into Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Difco Laboratories) at 28°C for 7-10 days (Stevenson et al., 2007). Bacterial growth was monitored using dark-field microscopy. The bacteria were centrifuged at 17000 rpm for 20 min, and the cell pellets were re-suspended in phosphate-buffered saline (PBS, pH 7.2). The cells were then inactivated by formalin 0.5% (v/v) and stored at 4ºC for 24 h. The cell suspensions contained 10-20 mg of cells (in terms of dry weight) per milliliter.

***Particle preparation.*** Alginate microspheres containing leptospiral antigen (LA) were prepared by a modified emulsification method (Jin et al., 2009). In short, LA was suspended in 10 ml of 2-5% (w/v) alginate solution and was mixed with 20 ml iso-octane containing 0.2-2% (w/v) Span 80. The mixture was emulsified for 3 min in a homogenizer (Heidolph, Germany) at 500-1000 rpm, followed by addition of 1.5 ml 3.75-30% (w/v) Tween 80 aqueous solution as the second emulsifier to achieve a suitable hydrophilic-lipophilic balance (HLB) value. The mixture was further stirred at the same speed for 3 min. Thereafter, 8 ml of 8% (w/v) calcium chloride solution was added drop-wise. This cross-linking process lasted for 3 min. After the mixture was stirred for another 3 min, the alginate microspheres were collected through centrifugation, washed twice with deionized water, and then the microspheres were suspended in 20 ml of 0.1% (w/v) poly-l-lysine. After homogenization for 10 min, the alginate microspheres were collected by centrifugation and washed with deionized water, and finally, dried in a vacuum desiccator.

***Particle characterization.*** The morphology of LA-loaded microspheres was observed using optical microscopy (BX51, Olympus Corporation, Japan). The detailed morphology of these microspheres was studied through scanning electron microscopy (SEM; Oxford, UK). Microspheres for SEM were mounted on metal stubs previously covered with double-sided adhesive and coated with gold in vacuum condition. Particle size (volume mean diameter) and size distribution of the microspheres were determined using a particle size analyzer (Malvern, UK).

***LE and LC of LA in alginate microspheres.*** LE 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. LC was estimated as the ratio between the mass of encapsulated agent in the recovered particles and the total mass of microspheres during particle production (Jin et al., 2009). After dissolving 80 g of dry particles in 5 mL of 0.75 M sodium citrate aqueous solution overnight (Lemoine et al., 1998), the samples were centrifuged at 10,000 rpm for 10 min, and the supernatant was neutralized by 0.1 M sodium hydroxide. Sample absorbance was determined at 585 nm and compared to a previously prepared standard curve. For each batch of microspheres, the LE and LC were determined in triplicates.

***In-vitro antigenicity of LA.*** A critical point in developing a carrier system for antigens is preservation of their native antigenicity. During alginate microsphere preparation, LA was exposed to potentially harsh conditions such as contact with surfactants and organic solvents. This may result in alteration of the structural stability and reduction of antigenicity of antigens. Thus, the in-vitro antigenicity of LA was measured with enzyme-linked immunosorbent assay (ELISA). Each sample was examined at different dilutions against a linear fitting to the responses of control standard samples with different dilutions (Shi et al., 2002). The in-vitro antigenicity of LA was

evaluated by the ratio of ELISA response and protein concentration (ELISA/protein).

***In-vitro release studies.*** The in-vitro release studies of alginate microspheres containing LA were carried out in PBS (pH 7.2). Accurately weighed amounts of LA-loaded alginate microspheres were put into Eppendorf tubes (about 20 tubes), with each tube containing 1 mg of the microspheres and 1 ml of PBS. The Eppendorf tubes were then incubated at 37 °C under continuous shaking for one week. At various time intervals, the supernatant (250 µl) was collected after centrifugation (13000 rpm for 10 min) and replaced with fresh medium. The released LA into the supernatant was quantified via Bradford protein assay method. The in-vitro release studies were performed in triplicates.

## *RESULTS*

***Effects of emulsion preparation conditions on particle characteristics.*** The impact of the preparation conditions such as the concentration of alginate solution, the concentration of emulsifiers, and the homogenization rate on the characteristics of the microspheres are shown in Tables 1-3. The results showed that the most influential parameter on the characteristics of the microspheres was the homogenization rate, followed by the concentration of alginate solution and the concentration of emulsifiers. The optimal parameters of the process were as follows: the concentrations of alginate solution and emulsifiers were 3.65% (w/v), Span 80 (0.24% w/v), and Tween 80 (3.85% w/v), respectively, and the homogenization rate was 500 rpm (Tables 1-3).

***Table 1.*** Effect of sodium alginate concentration on microsphere formation (3.75% w/v Tween 80, 0.2% w/v Span 80, and homogenization rate of 500 rpm)

| Alginate concentration ( % w/v ) | Yield of microspheres ( mg ) | Microsphere formation |
|---|---|---|
| 1.5 | 152±2 | Ag |
| 2.5 | 221±1.73 | Ag |
| 3.5 | 272±2.08 | P |
| 5 | 401.6±2.88 | D |

Ag: aggregation, P: proper microsphere, D: deformed microsphere

***Morphology, size, and particle size distribution.*** The obtained alginate microspheres and LA-loaded alginate microspheres were characterized by SEM and particle size analyzer. Two typical scanning electron microphotographs of blank microspheres and LA-loaded microspheres were presented in Figure 1.

***Table 2.*** Effect of concentration of emulsifiers on microsphere characteristics
(alginate concentration 3.5% w/v and homogenizing rate of 500 rpm)

| Span 80 ( % w/v ) | Tween 80 ( % w/v ) | Yield of microspheress ( mg ) | Emulsion stability and particle size |
|---|---|---|---|
| 0.1 | 2 | 229.66±2.51 | NS and P |
| 0.2 | 3.75 | 272±1.75 | S and P |
| 0.5 | 15 | 284.65±3.51 | S and IMP |
| 1 | 30 | 300±2.21 | S and IMP |

NS: nonstable emulsion, S: stable emulsion, P: proper microsphere, IMP: improper microsphere

***Table 3.*** Effect of homogenization rate on microsphere characteristics (alginate concentration 3.5% w/v, 3.75% w/v Tween 80, and 0.2% w/v Span 80)

| Homogenization rate (rpm) | Yield of microspheres (mg) | Emulsion stability and particle size |
|---|---|---|
| 500 | 274±1.53 | S and P |
| 700 | 252.66±2.07 | S and IMP |
| 1000 | 242±2.64 | S and IMP |

NS: stable emulsion, P: proper microsphere, IMP: improper microsphere

***Morphology, size, and particle size distribution.*** The obtained alginate microspheres and LA-loaded alginate microspheres were characterized by SEM and particle size analyzer. Two typical scanning electron microphotographs of blank microspheres and LA-loaded microspheres were presented in Figure 1. According to the figure, alginate microspheres were almost spherical (Figure 1A), and alginate microspheres containing LA were spherical without any aggregations (Figure 1B). The average size of alginate microspheres was about 189 µm and the mean size of LA-loaded alginate microspheres was approximately 200 µm, which was greater than that of blank microspheres. The size distribution of alginate

microspheres and LA-loaded alginate microspheres are presented in Figure 2, which indicated that well-dispersed alginate microspheres were prepared.

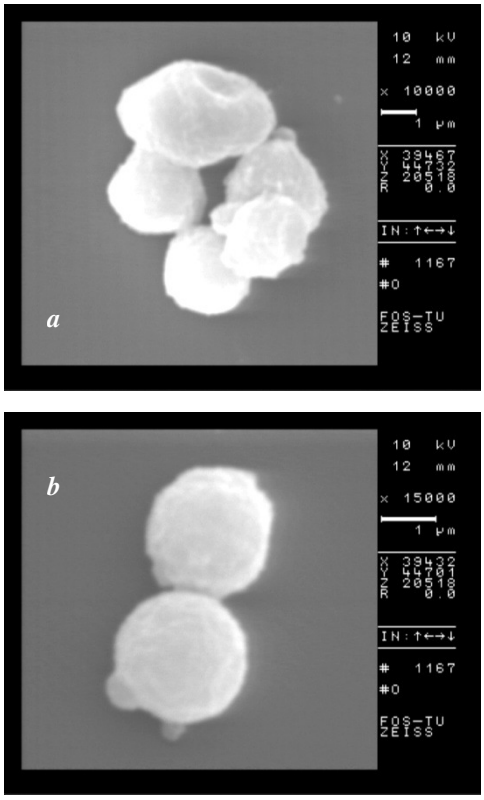


***Figure 1.*** Scanning electron microscopy images of alginate microspheres before loading (a) and after antigen loading (b) (sodium alginate concentration 3.5% w/v, 0.2 % w/v Span 80, 3.75% w/v Tween 80, and homogenization rate of 500 rpm).

***LE and LC of LA in alginate microspheres.*** To assess the ability of the alginate microspheres to incorporate the inactive bacteria, the effects of the initial LA content on LE and LC were evaluated. Figure 3 demonstrates the effect of LA concentration on the LE and LC of alginate microspheres. As shown in Figure 3, with increasing LA concentration, the LE and LC were increased. The LA with 20 mg/ml ($10^{10}$ cell/ml) concentration led to maximum LE (97.41%) and LC (8%). The alginate microspheres with concentration of 20 mg/ml of antigens were adapted to in-vitro release.

***In-vitro antigenicity.*** The antigenicity of the LA encapsulated in microspheres was evaluated by ELISA/protein ratio determination. Almost identical outcomes were observed for the native LA and the LA released from alginate microsphere (data not shown). There was no significant antigenicity loss (10%±2) induced by the encapsulation and subsequent release of LA from the alginate microspheres.

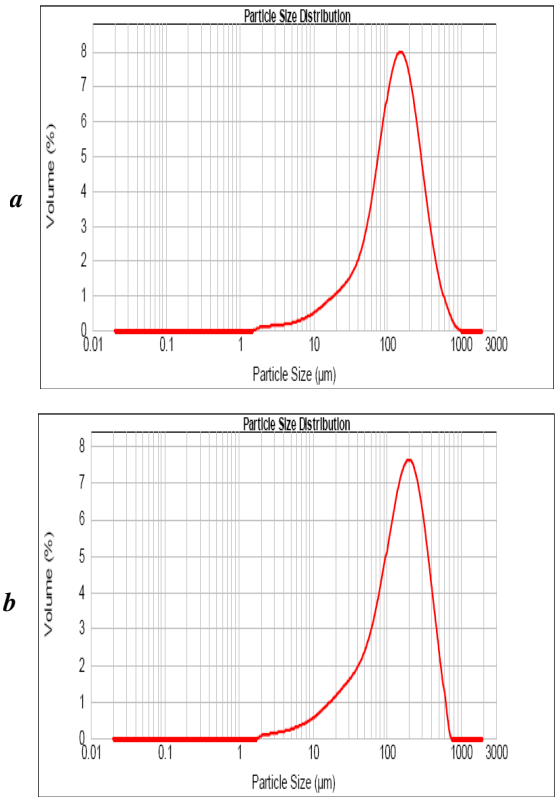


***Figure 2.*** Size distribution profile of alginate microspheres before loading (a) and after loading with antigens (b) (sodium alginate concentration 3.5% w/v, 0.2 % w/v Span 80, 3.75% w/v Tween 80, and homogenization rate of 500 rpm).

***In-vitro release.*** The in-vitro release of LA from the alginate microspheres was investigated in phosphate buffer solution (pH 7.2) at 37 °C. The slow release of LA from alginate microspheres occurred during the first eight hours, subsequent release was faster up to 48 hours and followed for the next 216 hours at an approximately constant rate. Roughly 64% of the LA released in five days (Figure 4).

## *DISCUSSION*

Leptospirosis, a zoonotic disease, is recognized as an important emerging infectious disease in the last 10 years. Prevention of leptospirosis without vaccination is impractical and difficult to achieve.

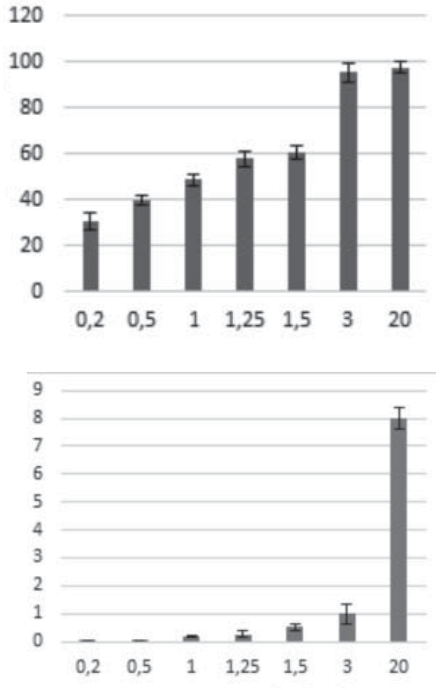


***Figure 3.*** Effects of different leptospiral antigen concentrations on loading efficiency (a) and loading capacity (b) of sodium alginate microspheres (sodium alginate concentration 3.5% w/v, 0.2 % w/v Span 80, 3.75% w/v Tween 80, and homogenization rate of 500 rpm).

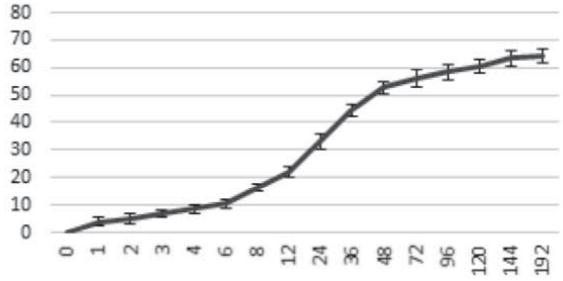


***Figure 4.*** In-vitro release profile of leptospiral antigen (LA) from alginate microspheres (sodium alginate concentration 3.5% w/v, 0.2 % w/v Span 80, 3.75% w/v Tween 80, homogenization rate of 500 rpm, and LA 20 mg/ml).

Currently available vaccines consist of killed whole-cell bacteria that are widely used in animals, but less so in humans (Adler and Moctezuma, 2009). The existing licensed vaccines suffer from several drawbacks including the required multiple doses to induce protective efficacy and repeated annual revaccination to retain immunity. Therefore, safe and efficacious immunization systems are needed to overcome these shortcomings. The hydrophilic microspheres have received considerable attention to deliver therapeutic peptides, proteins, and antigens by intravenous, oral, and mucosal administration. Alginates are widely applied in pharmaceutical studies as a carrier for drug and antigen delivery. Faisal et al. (2009a) showed that the variable region of recombinant Leptospira immunoglobulin such as protein A (LigAvar) incorporated into conventional liposomes and PLGA microspheres produced robust immune responses that induced significant protection against virulent L. interrogans serovar Pomona challenge in hamsters. This group in another study prepared novel liposomes from total polar lipids of non-pathogenic Leptospira biflexa serovar Potac (designated leptosomes) and evaluated their potential vaccine adjuvants with novel protective antigens (Lp0607, Lp1118 and Lp1454) of L. interrogans serovar Pomona in a hamster model. Their results demonstrated that leptosomes are better adjuvants than conventional liposomes as revealed by enhanced long-term antibody response, lymphocyte proliferation, and significant enhancement of both Th1 (IFN-γ) and Th2 (IL-4 and IL-10) cytokines (Faisal et al., 2009b). This study is the first attempt to produce biodegradable alginate microspheres for loading of leptospiral antigens and to evaluate their potential as delivery systems. Our results indicated that use of sodium alginate with concentrations lower than 3.65% w/v led to limited formation of mechanically resistant microspheres as a result of diluting the polymeric solution. Such microspheres were small, but during the production or drying processes, coalescence and aggregation were indicated due to high external energy. In contrast, the samples with sodium alginate concentrations of higher than 3.65% w/v were mostly deformed in shape and large in size. The probable reason was high viscosity of the polymeric solution.

Thereby, stirrer could not homogenize viscose aqueous phase in organic phase and produced deformed microspheres. These results were in accordance with those of the previous reports (Lemoine et al., 1998; Rodrigues et al., 2006; Tafaghodi et al., 2006), but they are in disagreement with the results reported by Cho et al. (1998). The discrepancy in the results might be due to employment of a higher homogenization rate in that study. Our study revealed that appropriate microspheres were fabricated with 0.24% w/v Span 80 and 3.85% w/v Tween 80. In line with the results of some other studies (Wan et al., 1994; Zheng et al., 2004; Jin et al., 2009), in lower concentrations of emulsifiers, the obtained emulsion was not stable and in their higher concentrations, the size of microspheres was considerably decreased as a result of reducing surface tension of dispersed phase droplets. Although one of the most influential parameters on the characteristics of microspheres was homogenization rate, application of proper stirring rates is of great significance. Based on evaluations in this study, stable and proper microspheres were made at a high stirring rate (500 rpm), which is congruent with the results reported by other studies (Cho et al., 1998; Rodrigues et al., 2006). The effects of LA concentration on LE and LC were investigated, and our results showed that by increasing LA concentration, LE and LC were enhanced. The LA with 20 mg/ml concentration led to maximum LE (97.41%) and LC (8%). Our results were similar to those of the majority of other experiments with alginate microspheres (Wan et al., 1992; Kim et al., 2002; Sangeetha et al., 2007). In the current study, to estimate particle size distribution and mean diameter of the alginate microspheres, normal particle size distribution before and after LA-loading were determined. The mean diameter of microspheres after LA-loading was increased. The enhancement of microspheres' diameter may be caused by electrostatic bounds between polymeric chains and antigen proteinic chains on the surface of microspheres. SEM images of LA-loaded microspheres showed that alginate microspheres were almost spherical without any aggregation, as was reported by other studies (Tafaghodi et al., 2006; Jin et al., 2009). Alginate matrixes were demonstrated useful for the slow release of several potential therapeutic proteins, and multiple studies demonstrated the efficacy of these systems (Tafaghodi et al., 2007). Similarly, in our study, the in-vitro release profile of LA-loaded microspheres showed the long release time and slow release rate of LA originated from the fact that LA macromolecules were bound onto microspheres by strong interactions. Additionally, after augmentation of poly-l-lysine, the electrostatic interaction between the positively charged -NH3+ of poly-l-lysine and negatively charged -COO- of alginate redounded to increase stability of microspheres. The presence of poly-l-lysine layer could reduce diffusion of LA from the microspheres and prolong the release time of LA. Subsequently, due to degradation and erosion of the matrix, LA might be released from the alginate microspheres in an extended profile. Our results are confirmed by Lemoine et al. (1998) and Anal et al. (2003). We concluded that the properties of the prepared microspheres containing leptospiral antigens were strongly dependent on concentrations of sodium alginate and emulsifiers, and the stirring rate of the emulsion was a critical factor for the preparation of proper microspheres. The optimum concentrations of alginate and emulsifiers led to formation of the desirable microspheres and consequently, high LE, LC, and sustained-release profile. Thus, the alginate microspheres containing leptospiral antigens could be used as an adjuvant for vaccination against leptospirosis.

### *Ethics*

I hereby declare all ethical standards have been respected in preparation of the submitted article.

### *Conflict of Interest*

The authors declare that they have no conflict of interest.

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