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PLGA Nanospheres Loaded with Autoclaved *Leishmania Major* (ALM) and CpG-ODN: Preparation and *in vitro* Characterization

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Abstract

Objective(s)
Several antigens, adjuvants and delivery systems have been evaluated for induction of protective immune responses against Leishmaniasis, but most of them have been inefficient. In this study, PLGA nanospheres as antigen delivery system CpG-ODN as an immunoadjuvant for increasing the immune responses against Autoclaved *Leishmania major* (ALM) were prepared and characterized.
Materials and Methods
PLGA nanospheres prepared by a double-emulsion (W/O/W) technique. The internal aqueous phase contained ALM and CpG-ODN, while the oily phase contained the solution of PLGA in dichloromethan and the external aqueous phase was PVA 7.5% (W/V) solution. Particulate characteristics were studied by scanning electron microscopy and particle size analysis. The encapsulation efficiency was determined by Lowry method for ALM and UV spectroscopy at 260 nm for CpG-ODN. The release profiles of antigen and CpG-ODN from nanospheres evaluated for one week.
Results
Nanospheres were spherical in shape, having smooth surfaces. Mean diameters for blank and ALM + CpG-ODN loaded nanospheres recorded as 302±129 and 300±128 nm respectively. Also, the encapsulation efficiencies of ALM and CpG-ODN were 71.6±8.8 and 49.1±2.4%, respectively. Evaluation of the release profiles of ALM and CpG-ODN from nanospheres showed that 44.8±0.8% of ALM and 29.5±0.2% of CpG-ODN released from nanospheres in one week.
Conclusion
The prepared nanospheres with desirable size, encapsulation efficiency, and slow rate of release, had acceptable features for future *in vivo* studies.

Keywords: *Leishmania major*, CpG-ODN, PLGA nanosphere, Vaccine

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Introduction

Leishmaniasis is a significant health problem in many parts of the world resulting in an estimated of 12 million new cases each year. Current treatment is based on chemotherapy, which is expensive, difficult to administer and becoming ineffective due to the emergence of drug resistance. Leishmaniasis is considered one of a few parasitic diseases likely to be controllable by vaccination. Extensive evidence from studies in animal models indicates that solid protection can be achieved by immunization with protein or DNA vaccines. However, to date no such vaccine is available despite substantial efforts by many laboratories (1). Recently, several immunoadjuvants like BCG, G-CSF (2,3) and CpG-ODN (4-7) and also, various delivery systems like PLGA microspheres (8) and liposomes (9-12) have been used to potentiate the immune response against leishmania antigens.

Biodegradable poly (D,L-lactic-co-glycolic acid) (PLGA) nanospheres and microspheres are promising delivery systems for protein, peptide and DNA vaccines (13,14). Nano- and microparticles could enhance the immune responses against their encapsulated antigen by various mechanisms. They could impart particulate nature to soluble antigens and increase their interaction with antigen presenting cells (APCs) and macrophages (13). They have also been shown to deliver peptide antigens to APCs (15) to generate Th1 type immune response even against poor immunogens (16,17). They could be used for co-encapsulation of antigen and adjuvant, to deliver them to the same APC and induction of higher immune responses compared with delivery of antigen and adjuvant in separate (13).

Oligodeoxynucleotides (ODN) containing unmethylated CpG motifs act as immune adjutants and induces strong humoral and cellular immune responses with a bias towards a T helper type 1 (Th1) response (18-20). There are data demonstrate 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 (21-23).

The immunogenicity of the antigen and potency of the adjuvant could be substantially enhanced by co-delivery in biodegradable microspheres (13).

In another study, CpG ODN was co-encapsulated with TT in PLGA nanospheres. The immunoadjuvant potential of CpG-ODN on cellular immunity increased several fold (compared with injection of CpG ODN in solution), while the immunopotentiation effect of the particulate delivery on antibody response (total IgG and subtypes) was not so marked (24).

The efficiency of PLGA nanospheres in delivery of encapsulated TT and CpG ODN to dendritic cells (DCs) to achieve antigen specific T cell activation was also studied by Diwan et al. (25). The DCs pulsed with TT and CpG ODN together in nanoparticles induced significantly higher T cell proliferation ($P<0.05$) as compared to when DCs pulsed with TT and CpG ODN in solution. These results indicate that PLGA nanoparticles mimicking certain features of pathogens are efficient delivery systems for targeting vaccine antigens to DCs and activation of potent T cell responses (25).

Therefore, the aim of this study was to prepare and characterize PLGA nanospheres encapsulated with autoclaved leishmania major (ALM) and CpG-ODN adjuvant for immunization against leishmaniasis. Nanospheres will be injected to Balb/c mice for evaluation of their immune induction potential.

Materials and Methods

Materials

PLGA 50:50 co-polymer (MW 30000) was purchased from Boehringer Ingelheim (Germany). CpG oligodeoxynucleotide (# 1826, seq (5′-3′): tccatgacgttcctgacgtt) was purchased from Microsynth (Switzerland). Dichloromethane was from KianKaveh (Iran), acetonitrile and chloroform was from Merck (Darmschtadt, Germany). Bovine serum

albumin (BSA), Folin reagent and polyvinyl alcohol (PVA) (87–89% hydrolyzed, MW 31000-50000 g/mol) were obtained from Fluka (Buchs, Switzerland). Autoclaved *Leishmania major* (ALM) was provided by Razi Inst. (Hesarak, Karaj, Iran).

Methods

Preparation of PLGA nanosphere encapsulated with ALM and CpG-ODN

Nanospheres were prepared using a W/O/W emulsion and solvent evaporation technique (26). Briefly, CpG-ODN (40 µl, 10 µg/µl) and ALM (110 µl, 70 µg/µl) solutions were mixed and emulsified with PLGA solution (600 µl, 33% w/v) in dichloromethane for 40 s using a microtip probe sonicator (MSE, England) in amplitude 18. Ice-water bath was used for prevention of temperature rise in sonication processes. The W/O emulsion was then combined with PVA solution (8 ml, 7.5% w/v) and sonicated (80 s) to form the W/O/W emulsion. The secondary emulsion was then added to PVA solution. The emulsion was further stirred for 2 hr Nanospheres were collected by centrifugation (20000 g, 15 min, 4 ^{o}C) washed twice with distilled water, and then lyophilized.

Morphology and Particle size determination

Scanning electron microscope (Leo, Germany) was used for studying the morphology of nanospheres. For this purpose, nanospheres were coated with gold and palladium by a sputter coater (SC7620, Germany). Particle size and size distribution of the nanospheres was determined using a laser diffraction size analyzer (Shimadzu, Japan).

Encapsulation efficiency of ALM and CpG-ODN in PLGA nanospheres

The ALM and CpG-ODN content of PLGA nanospheres was determined using a 'two-step' extraction method (27). Briefly, known amounts of freeze-dried nanospheres were dissolved in dichloromethane (DCM, 1 ml). The suspension of dissolved polymer and precipitated ALM and CpG-ODN was centrifuged at 10000 g for 10 min and the polymer containing supernatant was then discarded. The pellet consisting of the precipitated encapsulates was redispersed in 0.1 N NaOH and the amount of ALM was determined by the Lowry protein assay method (28). Amount of CpG-ODN was estimated spectrophotometrically based on absorbance at 260nm (29). As ALM has absorbance at 260 nm, it was not possible to quantify them simultaneously. Therefore, nanospheres encapsulated with ALM or CpG-ODN were prepared under similar conditions and were used to estimate the encapsulation efficiencies, separately. For each batch of nanospheres the encapsulation efficiency was determined in triplicates.

In vitro release studies of ALM and CpG-ODN

PLGA nanospheres (40 mg) containing ALM were suspended in phosphate buffered saline (PBS, 600 µl, 10 mM, pH 7.4, containing 0.01% sodium azide) for 1 week. The suspension was gently shaken in a water bath at 37 ^{o}C. At various time intervals, the supernatant (500 µl) was removed after centrifugation (10000 g for 10 min) and replaced with fresh medium. ALM released into the supernatant was quantified by Lowry method. Similarly, CpG-ODN containing nanospheres were incubated in PBS buffer and the released fraction was estimated spectrophotometrically based on absorbance at 260 nm (29).

Statistical analysis

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

Results

Morphology and Size characteristics of PLGA nanospheres

As shown in Figure 1, spherical nanospheres with smooth surfaces were obtained. Addition of encapsulates has not affected the morphology and surface roughness of nanospheres.

The mean diameter of blank nanospheres or nanospheres encapsulated with ALM and CpG-ODN was 302±129 and 300±128, respectively. The mean diameter of both kinds of nanospheres was similar.

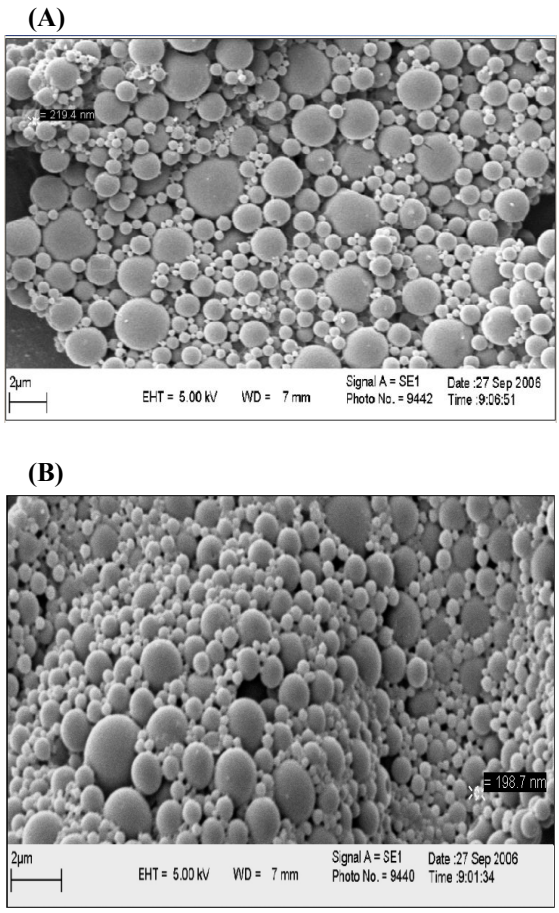

Figure 1. Scanning electron micrograph of blank PLGA nanospheres (A) and nanospheres encapsulated with ALM + CpG-ODN (B).

Encapsulation efficiency of ALM and CpG-ODN in PLGA nanospheres

The mean encapsulation efficiencies of ALM and CpG-ODN in nanospheres were 71.6±8.8 and 49.1±2.4%, respectively. The encapsulation rate of ALM was significantly higher than the CpG-ODN ($P < 0.05$).

In vitro release profiles of ALM and CpG-ODN from PLGA nanospheres

The release profile of ALM and CpG-ODN from nanospheres was evaluated (Figure 2). The initial release of ALM was seen in the first 2 hr of the study, in which 33.1±0.7% of the ALM was released. This initial burst release was followed by a plateau with a mild slope. Finally, after 1 week the cumulative percent of released ALM reached to 44.8±0.8%. In the case of CpG-ODN, the same pattern of release was seen. The initial burst release in the first 2 hr was 36.9±2%, and reached to 48.1±0.1% after 1 week. While it was expected that the release of smaller molecule (CpG) be faster than bigger one (ALM), the ALM showed higher initial and total release.

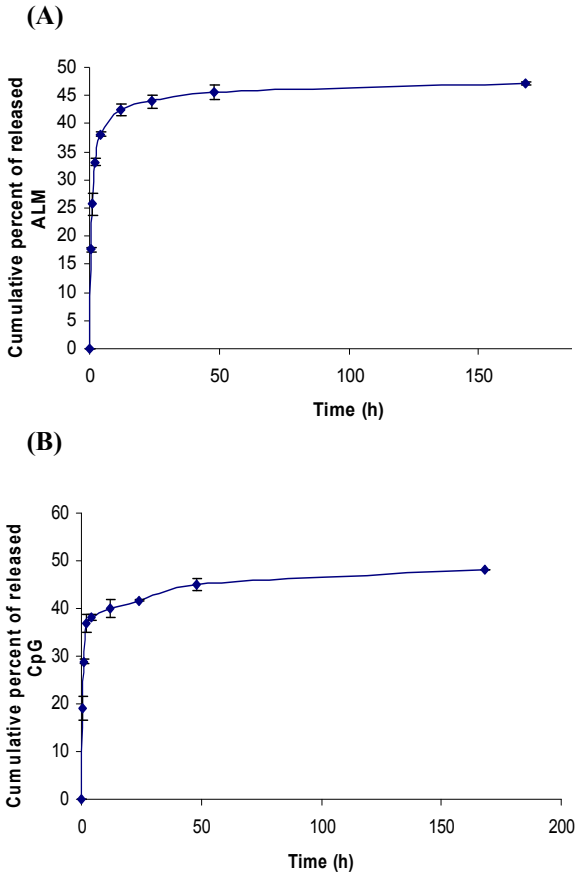

Figure 2. In vitro release of encapsulated ALM (A) and CpG-ODN (B) from PLGA nanospheres. Nanospheres were suspended in PBS buffer at 37 ºC under continuous shaking. At various times the cumulative amounts of released ALM (A) and CpG-ODN (B) were quantified. Error bars represents the SD (n=3)

Discussion

The mean diameter of particulate systems is an important factor in their efficient interaction with immune cells. Particles smaller than 10 µm in diameter, could be directly taken up by macrophages and dendritic cells through phagocytosis. This is an important property for stimulating the immune system, whereas larger microspheres (greater than 10 µm) need to undergo biodegradation, before phagocytosis

can occur (30). Degradation, antigen release, location and antigen presentation of microspheres smaller than 10 μm are expected to be different from larger ones (30).

The particle size of spheres could be adjusted by various parameters. The process of nanosphere preparation is one of the determinative factors in particle size (31). Gutierro and his co-workers have prepared 3 sizes (200 nm, 500 nm and 1000 nm) of PLGA nanospheres by solvent extraction method. They changed the mechanical instruments (homogenizer for larger particles and sonicator for smaller ones) and concentration of PVA in outer aqueous phase (32). Of the other parameters which could affect the particle size are viscosity of internal phase of emulsion, encapsulate to polymer ratio and volume of internal and external aqueous phases (33).

In this study the above mentioned parameters were adjusted. based on previous studies (13, 34, 35), The mean diameter of blank nanospheres or nanospheres encapsulated with ALM and CpG-ODN was 302±129 and 300±128, respectively. The mean diameter of both kinds of nanospheres is similar. This could be related to small amount of encapsulates which was added in formulation. In another study, we prepared nanospheres encapsulated with tetanus toxoid (TT) with the same method. The mean diameter of nanospheres was 290.6 nm (13). For cancer vaccination studies, we encapsulated the mucin 1 lipopeptide (MUC1) and CpG-ODN in PLGA nanospheres. The mean diameters of nanospheres encapsulated with MUC1 and MUC1 + CpG-ODN were 650 and 518 nm (34). PLGA nanospheres were prepared by the same method as described above and the only difference was the solvent system of the oil phase of emulsion which was a mixture of chloroform and methanol due to the lipophilic nature of MUC1, while in the present study dichloromethane was used. The differences between diameters could be resulted from different solvent systems used.

In particulate delivery systems, encapsulation efficiency of encapsulates is a determinant factor, because larger encapsulation efficiency can prevent the loss of encapsulate and also limits the need of administering high level of carrier (36).

The encapsulation efficiency could be affected by several parameters. Increasing the viscosity of internal aqueous phase will enhance the encapsulation efficiency. But it usually results in larger particles (33). Increasing the viscosity of external aqueous phase will also increase the encapsulation efficiency (37). Antigen to polymer weight ratio and lactide to glycolide ratio in PLGA polymer are also among other important factors (33). Larger volumes of internal and external aqueous phases will increase the encapsulation rate. Higher volumes of external aqueous phase will simultaneously increase the particle size (33, 38). The higher molecular weight and solubility of encapsulate could also increase the encapsulation efficiency (30, 39).

In this study higher viscosity of external aqueous phase (7.5% w/v PVA in water) was used to increase the encapsulation efficiency. The mean encapsulation efficiencies of ALM and CpG-ODN in nanospheres were 71.6 ± 8.8 and 49.1 ± 2.4%, respectively. The difference between ALM and CpG-ODN encapsulations ($P<0.05$), could be related to higher MW of ALM. In another study, PLGA nanospheres encapsulated with tetanus toxoid (TT) and CpG-ODN were prepared by the same method. The encapsulation efficiencies were of TT and CpG-ODN were 73.5% and 53.3%, respectively which are similar to those found in this study (13). The same method was also used for encapsulation of MUC1 antigens in PLGA nanospheres (34) and the encapsulation efficiency of MUC1 lipopeptide was calculated as 30% (34). The lower encapsulation of MUC1, compared with ALM, could be attributed to its lower molecular weight and water solubility (30, 39).

The optimum release profile for each particulate delivery system depends on purpose of the study. The release profile of various encapsulates from PLGA microspheres and nanospheres have been widely studied. In this study, PLGA nanospheres have been used for encapsulation of ALM antigen and

CpG-ODN adjuvant. It has been shown that the particulate antigens have better interaction with antigen presenting cells and macrophages and could induce higher immune responses, compared with soluble antigens (19, 40). So, in this study nanospheres have been used to convert the soluble antigens to particulate ones. In such a study, because of better immune stimulation potential of encapsulated antigens, the best release rate is the lowest one.

The impact of various parameters on the release profile from PLGA microspheres and nanospheres has been evaluated. Glycolide to lactide ratio and molecular weight of co-polymer (41-43), polymer crystalinity (39) are of the most important parameters. Additionally, presence of the small pores on the surface of the spheres (42) and concentration of the PVA on the surface of the spheres (37) could affect the profile and the total release of encapsulate.

In this study the initial release of ALM was seen in the first 2 hr of the study, in which 33.1±0.7% of the ALM was released. The initial burst release was followed by a plateau with a mild slope. Finally, after 1 week the cumulative percent of released ALM reached to 44.8±0.8%. In the case of CpG-OD,N burst release in the first 2 hour was 36.9±2%, and reached to 48.1 ± 0.1% after 1 week. While it was expected that the release of smaller molecule (CpG) be faster than bigger one (ALM), the ALM showed higher initial and total release. Release profile from microspheres could be affected by the antigen to polymer ratio (44). Higher ratio could increase the initial and total release (45). At the present study, each milligram of nanospheres contained 18 μg ALM or 1 μg CpG-ODN. The antigen to polymer ratio for ALM is 18 times higher than the CpG-ODN. So despite the higher MW of ALM, its release was higher. In another study, we encapsulated TT and CpG-ODN in PLGA nanospheres with the same method and their release profiles were studied similarly (13). The initial burst release of TT was 9.6±2% in the first hour and reached to about 15% in the first 1 week. CpG-ODN showed a burst release of about 20% in the first hour, and reached to about 40% after 1 week. In that study, in presence of the nearly identical antigen to polymer ratio for CpG and TT, the larger molecules (TT) have shown less initial and total release. In another study the effect of protein molecular weight on release kinetics from polymeric microspheres (1-3 micron) was evaluated. Proteins were encapsulated at high and low loadings (protein to polymer ratio) in PLGA microspheres. Mechanism of release from microspheres appeared to be dependent on protein molecular weight for microspheres with low loadings (0.5-1.6%), while independent of protein molecular weight for microspheres with high loadings (4.8-6.9%). At low loadings, release of larger proteins was dependent on diffusion through pores for the duration of the study, while smaller proteins seemed to depend on diffusion through pores initially and on degradation at later times (46).

Conclusion

The present study demonstrated that PLGA nanospheres with optimum characteristics can be prepared by solvent extraction/evaporation method. Nanospheres had a mean diameter of about 300 nm. A reasonable encapsulation efficiency for ALM (71.6±8.8) and CpG-ODN (49.1±2.4%) 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. All these characteristics make PLGA nanospheres encapsulated with autoclaved leishmania major (ALM) and CpG-ODN adjuvant, particularly interesting for immunization against leishmaniasis.

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