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Preparation and evaluation of niosomes containing autoclaved *Leishmania major*: a preliminary study

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Abstract

In this study, different positively charged niosomal formulations containing sorbitan esters, cholesterol and cetyl trimethyl ammonium bromide were prepared by film hydration method for the entrapment of autoclaved *Leishmania major* (ALM). Size distribution pattern and stability of niosomes were investigated by laser light scattering method and ALM encapsulation per cent was measured by the bicinchoninic acid method. Finally, the selected formulation was used for the induction of the immune response against cutaneous leishmaniasis in BALB/c mice. Size distribution curves of all the formulations followed a log-normal pattern and the mean volume diameter was in the range 7.57–15.80 μm. The mean volume diameters were significantly increased by adding Tween to Span formulations ($p < 0.05$). The percentage of ALM entrapped in all formulations varied between 14.88% and 36.65%. In contrast to ALM, *in vivo* studies showed that the niosomes containing ALM have a moderate effect in the prevention of cutaneous leishmaniasis in BALB/c mice.

Keywords: Sorbitan esters, cholesterol, non-ionic surfactant vesicles, cutaneous leishmaniasis

Introduction

A number of new vaccines containing protein subunits or synthetic peptides exhibit low immunogenicity and so, to increase the immune response, the adjuvants are required (Conacher et al., 2000a). The most common types of adjuvants used in recent years are aluminium compounds (Alum). Such adjuvants mainly stimulate humoral immune response and cannot effectively induce cell-mediated immune responses (Conacher et al., 2000a; Lindblad, 2004). Furthermore, the alum compounds have shown some unwanted side effects. Consequently, extensive research have been done to produce and exploit the new adjuvants that lead to the use of vesicular systems for the clinical study of influenza–tetanus–diphtheria liposomal vaccine (Gregoriadis et al., 1996) and hepatitis A liposomal vaccine (Epaxal-Berna®) (Ambrosch et al., 1997).

Liposomes were composed of phospholipids producing special closed-layer membranes in aqueous media and they entrapped the materials in vesicles and between the lipid layers (Christensen et al., 2011). Liposomes showed poor adjuvant activity which is more pronounced for antigens with low immunogenicity (Henriksen-Lacey et al., 2011).

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Table 1. Composition of niosomes containing ALM, ALM encapsulation efficiency (EE%) and mean volume diameter of vesicles at different time intervals after preparation.

Surfactant name	Molar ratio				ALM EE% ± SD	Mean volume diameter (μm) ± SD	
	Surfactant		Cholesterol	CTAB			
	S	T				72 h	3 months
S 20	6	0	3	1	36.65 ± 1.00	9.87 ± 0.14	59.90 ± 0.32
S 40	6	0	3	1	32.81 ± 1.84	10.71 ± 0.38	14.30 ± 0.56
S 60	6	0	3	1	27.37 ± 1.28	9.64 ± 0.17	9.93 ± 0.33
S 80	6	0	3	1	27.20 ± 0.88	8.54 ± 0.54	40.20 ± 0.56
T 20	0	6	3	1	14.88 ± 2.60	8.06 ± 0.53	11.24 ± 0.48
T 40	0	6	3	1	26.83 ± 1.40	7.83 ± 0.07	10.13 ± 0.46
T 60	0	6	3	1	28.84 ± 1.80	11.70 ± 0.16	44.47 ± 0.53
T 80	0	6	3	1	15.08 ± 1.76	7.57 ± 0.10	10.35 ± 0.51
S 20/T 20	3	3	3	1	26.61 ± 0.96	11.33 ± 0.15	10.93 ± 0.32
S 40/T 40	3	3	3	1	33.01 ± 1.60	10.80 ± 0.98	11.50 ± 0.57
S 60/T 60	3	3	3	1	33.28 ± 0.84	12.25 ± 0.20	13.08 ± 0.39
S 80/T 80	3	3	3	1	29.69 ± 2.80	9.43 ± 0.13	9.10 ± 0.28

Notes: S, Span; T, Tween; and CTAB, cetyl trimethyl ammonium bromide; EE%, percent of encapsulation efficiency. Mean ± SD, $n = 3$.

Some phospholipids are susceptible to oxidation in the presence of air. Hence, manufacturing, packaging and storing of liposomes must be done in the presence of an inert gas such as nitrogen (Jesorka and Orwar, 2008). Consequently, the use of sustainable alternatives such as non-ionic surfactants is inevitable, especially for creating a strong cellular immune response against intracellular organisms (Perrie et al., 2008). Niosomes (non-ionic surfactant vesicles (NISV)) are resistant to oxidation and do not require special packaging and storing conditions (Karim et al., 2010). Compared to liposomes, niosomes have some advantages such as chemical stability, lower costs of the chemicals and the large amount of surfactant classes available to design these vesicular systems (Jain and Vyas, 2006; Huang et al., 2011). Previous studies on the adjuvant properties of niosomes exhibited that the niosomes containing bovine serum albumin (BSA) caused a stronger immune response than BSA formulated in Freund's complete adjuvant (Brewer and Alexander, 1992). The soluble antigens of *Toxoplasma gondii* formulated in a niosomal system produced strong cellular immune response against toxoplasmosis (Roberts et al., 1994).

Leishmaniasis comprises a wide range of diseases from self-limited skin diseases to diffuse or severe visceral diseases caused by different species of the genus *Leishmania* (Reithinger et al., 2007). Cutaneous leishmaniasis is an important health problem and many efforts have been conducted to achieve effective vaccines (Murray et al., 2005; Mishra et al., 2007).

According to the application of autoclaved *Leishmania major* (ALM) in vaccination against cutaneous leishmaniasis (Misra et al., 2001; Rhee et al., 2002), the aim of this study was to formulate positively charged niosomes containing ALM and to evaluate their physicochemical characteristics. In the next step, the *in vivo* performances of the selected formulation were examined in order to make use of the properties of this new adjuvant against cutaneous leishmaniasis.

Materials and methods

Materials

Non-ionic surfactants including sorbitan monolaurate (Span 20), sorbitan monopalmitate (Span 40), sorbitan monostearate (Span 60), sorbitan monooleate (Span 80), polysorbate 20 (Tween 20), polysorbate 40 (Tween 40), polysorbate 60 (Tween 60) and polysorbate 80 (Tween 80) as well as RPMI 1640 medium and foetal calf serum (FCS) were purchased from Sigma–Aldrich (St. Louis, MO). Cholesterol (Chol) and cetyl trimethyl ammonium bromide (CTAB) were supplied by Fluka (Switzerland). The ALM (7.5×10^8 promastigote per mL, 11.11 mg/mL of protein) produced from promastigotes of *L. major* (MRHO/IR/76/ER) was purchased from the Razi Vaccine and Serum Research Institute, Hesarak, Iran. All other chemicals and solvents were of analytical grade.

Preparation of NISV

For the preparation of niosomes, a modified version of lipid hydration method was used as previously described (Pardakhty et al., 2007). Briefly, various non-ionic surfactant/Chol/CTAB ratios were used to prepare formulations (Table 1). In this method, 150 μmol of each mixture were dissolved in 10 mL chloroform in a 100-mL round-bottomed flask. To form a thin film on the wall of the flask, the organic solvent was removed under reduced pressure (60°C, 120 rpm) by a rotary evaporator (Buchi, Switzerland). Residual chloroform was evaporated in a vacuum oven for 8 h at room temperature followed by the

hydration of the dried film using 5 mL isotonic phosphate buffered saline (PBS) (pH 7.2) containing ALM (500 μg/mL of protein) with a mild rotation in water bath at 60°C for 30 min. The resulting multi-lamellar NISV dispersions were left to gradually cool and stored at 4°C.

Morphology and size distribution of prepared vesicles

To evaluate the formation and morphology of vesicles, the suspensions were observed by optical microscope (Zeiss, Germany) equipped with a computer-controlled image analysis system in 10×40 and 10×100 magnifications. In this study, 72 h after preparation, the particle size distribution patterns of various formulations containing ALM were determined by static laser light diffraction method in a particle analyzer (Malvern Master Sizer X, Malvern, UK). Measurement was carried out using a 100 mm focal length lens, which was capable of measuring vesicles in the 0.5–180 μm size range. The fundamental size distribution derived by this technique was volume based and the size distribution was expressed in terms of the volumes of equivalent spheres (dv). Size distribution parameters and derived diameters were calculated from the fundamental size distribution using Sizer-X software.

ALM encapsulation efficiency

To separate non-entrapped ALM, the vesicle suspensions were centrifuged (90 XL Ultracentrifuge, Beckman, USA) at $11\,000 \times g$ for 30 min at 4°C and washed twice with PBS (pH 7.2). After disrupting the niosomes by isopropyl alcohol, the amounts of ALM in the supernatant and in the pellets were measured by Micro BCA™ Protein Assay Kit (Pierce, Rockford, IL) using a BSA standard curve. ALM encapsulation efficiency (EE) was calculated as follows:

$EE\% = 100 \times$ (amount of incorporated ALM/amount of ALM used for vesicle preparation)

Physical stability of prepared niosomes

Aggregation or fusion of the vesicles as a function of storage duration was determined as the changes in vesicle diameter by laser light scattering method. The vesicles were stored in glass vials in refrigerator (4°C) for 3 months. The changes in morphology of niosomes and also the component separation were evaluated by the optical microscope. No special precautions were taken to improve the stability of vesicles.

Animals and treatments

In this study, 32 male BALB/c mice (body weight 20–22 g) were used. The mice were purchased from the Pasteur Institute of Iran (Tehran, Iran) and housed in plastic cages in a colony room with controlled temperature (22 ± 1°C) and humidity (50 ± 10%) and a 12/12 h light/dark cycle. The mice had free access to tap water and food. The experimental procedures carried out in this study were in compliance with the guidelines of the Kerman University of Medical Science (Kerman, Iran) for the care and use of laboratory animals. The mice were divided into control, ALM, niosomes without ALM and niosomes containing ALM groups and were subcutaneously vaccinated by 0.1 mL of PBS (pH 7.2), ALM (50 μg of protein/mouse), niosomes without ALM and niosomes containing ALM (50 μg of protein/mouse), respectively. To vaccinate the mice, preparations were inoculated three times at 2-week intervals. *Leishmania major* (MRHO/IR/76/ER) were cultured in RPMI 1640 medium supplemented with 10% (v/v) FCS at 27°C in a CO_2 incubator (5% CO_2 and 95% relative humidity). Four weeks after the last immunization, the BALB/c mice were subcutaneously injected at the base of tail with 0.1 mL culture media containing 1×10^6 stationary phase promastigotes. Finally, the mean diameters of lesions were measured weekly.

Statistical analysis

Each value was expressed as the mean ± SD. Software SPSS 15 for windows (SPSS Inc., Chicago) was used for statistical analysis. The differences between groups were determined using one-way analysis of variance test and *p*-values less than 0.05 were considered significant.

Results and discussion

The adjuvant activities of different non-ionic surfactants such as Tween 80, Span 85 (Vogel and Powell, 1995) and Span 40 and 60 (Chattaraj and Das, 2003) have been previously reported. Due to low toxicities of these compounds (Conacher et al., 2000b), sorbitan esters (Span) and their polyoxyethylene derivatives (Tween) were selected for preparation of niosomes containing ALM. The ability of surfactants to shape vesicles depends on the properties of the surfactant such as balance between hydrophilic surface area, the volume of the hydrocarbon chain and the maximum length that the chains can assume (Manosroi et al., 2003). All used surfactants formed niosomes in the presence of ALM. Niosomes were uniform in size and approximately spherical in shape. The micrographs in Figure 1 exhibit the formation of vesicular structures.

The presence of hydrophobic compounds with high molecular weight such as cholesterol is essential for the formation and stabilization of two layers in niosomes (Baillie et al., 1985; Uchegbu and Vyas, 1998). Although the presence of charge-inducing agents leads to the uniformity and stability of the vesicles, some negatively charged inducing compounds such as dicetyl phosphate exhibit low adjuvant activities (Conacher et al., 2000a). Distribution of CTAB in bilayers may increase the curvature of bilayers through electrostatic repulsion between the ionized head group, thus increasing the hydrophilic surface area and this

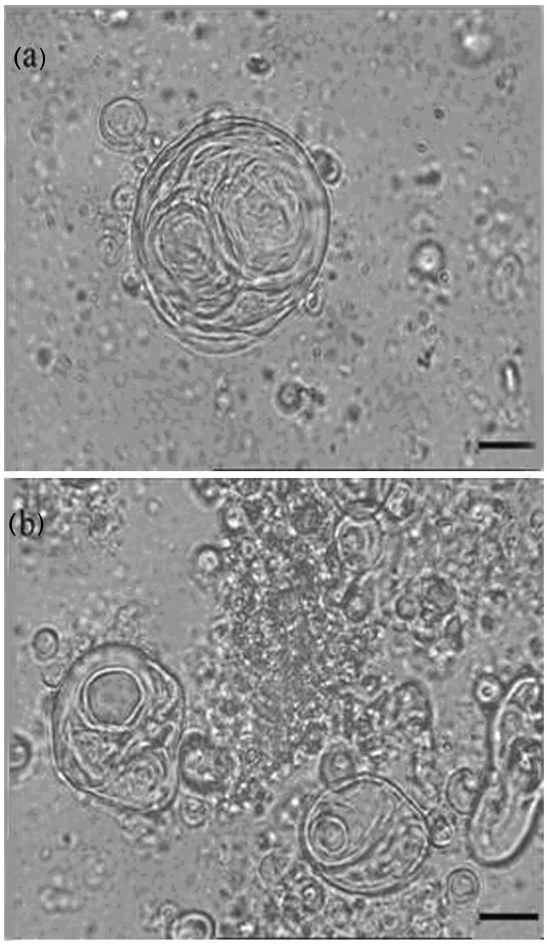

Figure 1. Optical micrographs (×1000 magnification) of ALM-containing niosomes prepared by film hydration method. Niosomes were composed of (a) Span 20/Chol/CTAB (6:3:1 molar ratio) and (b) Span 40/Tween 40/Chol/CTAB (3:3:3:1 molar ratio), Scale bar = 10 μm.

effect will result in smaller vesicles. Vesicles containing positive charge lead to cell-mediated immune response (Nakanishi et al., 1997). This effect is very important for immunity against intracellular pathogens such as *L. major*. Thus, in this study, CTAB was employed as a positively charged inducing agent.

The particle sizes of different formulations are presented in Table 1. All formulations demonstrated log-normal particle size distribution patterns. The smallest size ($7.57 \pm 0.10\,\mu m$) and the biggest size (12.25 ± 0.20) were observed in the Tween 80/Chol/CTAB and Span 60/Tween 60/Chol/CTAB vesicles, respectively (Table 1). The mean volume diameters were significantly increased by adding Tween to Span formulations ($p < 0.05$).

With the exception of span 20/Chol/CTAB formulation, the mean volume diameter of niosomes increased by increasing in the hydrophilic–lipophilic balance (HLB) value from Span 80 (HLB 4.3) to Span 60 (HLB 4.7) and Span 40 (HLB 6.7) (Table 1). The same result was reported about niosomes composed of sorbitan monoesters (Span 20, 40, 60 and 80) or a sorbitan triester (Span 85). These results might be anticipated since surface-free energy decreases with the increase in hydrophobicity (Yoshioka et al., 1994).

The change in particle size as a major indicator for niosome stability during storage at 4°C for 3 months was exhibited in Table 1. Furthermore, precipitation of the membrane components was evaluated by microscopic observation. Lawrence et al. (1996) suggested that size increment was due to fusion of the membranes and growth rather than vesicle aggregation. To avoid this effect, in our study, we used electrostatic repulsion among vesicles using the positively charged surfactant. Niosomes prepared from solid-state surfactants (Span 40 and 60), exhibited lower change in particles size and were morphologically more stable than Span 20 and 80 formulations under storage conditions. These significant alterations in stability markers may result from the fluidity of Span 20 and 80 bilayers leading to more leaky and unstable structure. Microscopic observation also revealed the formation of the needle-like and plate crystals in these formulations. This effect was previously reported about the niosomes containing insulin and tretinoin prepared by Span 80 (Manconi et al., 2002; Varshosaz et al., 2003). Except for Span 60, which was stable after 3 months, addition of Tweens to Span formulations significantly improved the particle size stabilities of all formulations during storage conditions ($p < 0.05$). This stability may be due to steric stabilization following surface modification of niosomes by polyoxyethylene derivatives. As an example, the size distribution curves of niosomes containing Span 20/Chol/CTAB and Span 20/Tween 20/Chol/CTAB after 72 h and 3 months were shown in Figure 2(a) and (b), respectively.

The niosome components play a critical role in EE% of protein antigens. EE% of influenza antigens formulated in Span 40 or 60/Chol/dicetyl phosphate was between 14% and 23% (Chattaraj and Das, 2003). Furthermore, for BSA and haemagglutinin formulated in niosomes prepared by Span 60, Chol and Solulan C24, the reported EE% was 30% (Murdan et al., 1999). ALM is composed of various soluble and insoluble compounds. Thus, it should be able to enter into a vesicular structure both in the lipophilic bilayer and in the hydrophilic core. The percentage of ALM entrapped in all niosome formulations varied between 14.88% and 36.65% (Table 1). Tween 20/Chol/CTAB niosomes had the lowest amount of entrapped ALM (Table 1). This effect may be due to these facts that surfactants with HLB < 10 can better shape niosomes than other surfactants (Varshosaz et al., 2005) and Tween 20 had the highest HLB among the surfactants used in this study (HLB 16.7). Except for Tween 20 formulation, the EE% in Tween 80/Chol/CTAB is significantly lower than other niosomes ($p < 0.05$) (Table 1). The alkyl chain in Tween 80 is unsaturated, which leads to leaky bilayers in niosomes and low EE%. No significant difference was observed between EE% data for the niosomal formulations composed of Span/Chol/CTAB and Span/Tween /Chol/CTAB ($p > 0.05$). Formerly insufficient effect of using ALM with BCG at single dose against leishmaniasis was reported (Sharifi et al., 1998). Thus, the researcher used other methods for

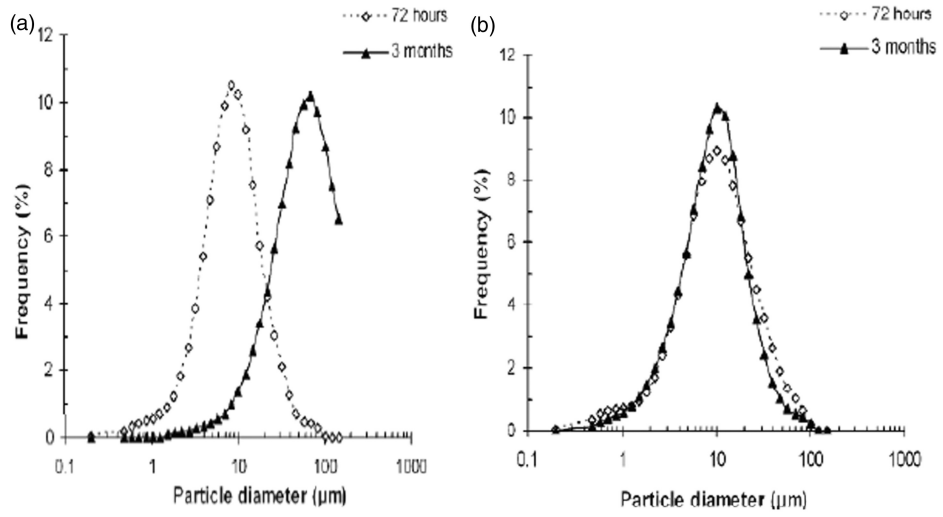

Figure 2. The vesicle size distribution of ALM-containing niosomes at different time intervals after preparation. Niosomes were composed of (a) Span 20/Chol/CTAB (6:3:1 molar ratio) and (b) Span 20/Tween 20/Chol/CTAB (3:3:3:1 molar ratio).

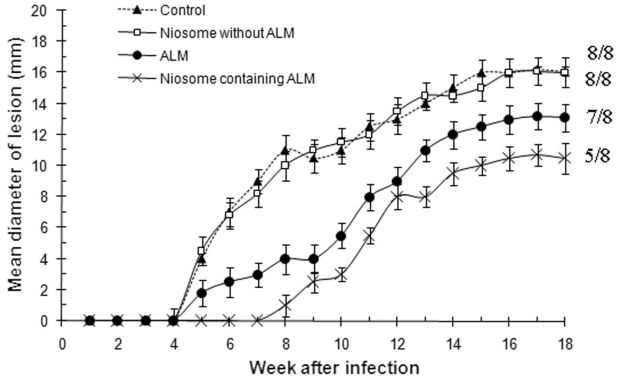

Figure 3. The effect of subcutaneous vaccination by PBS (pH 7.2) (control group), ALM, niosomes without ALM and niosomes containing ALM against cutaneous leishmaniasis in BALB/c mice.
Note: Data are expressed as the mean ± SD, ($n = 8$), the number of mice which developed lesions is indicated after each line.

vaccination such as the use of multiple doses of ALM with BCG vaccine (Alimohammadian et al., 2002) and the combination of ALM with interleukin-12 (Kenney et al., 1999). In this study, we employed multiple doses of ALM entrapped in positively charged niosomes for stimulating the immune response against cutaneous leishmaniasis in mice. Based on the physicochemical studies, the Span 40/Tween 40/Chol/CTAB formulation showed enough EE% and particle size stability was preferred for *in vivo* experiment. The results of *in vivo* studies were shown in Figure 3.

In control and niosomes without ALM group, the lesions were progressively developed after 4 weeks and all animals showed lesions after 18 weeks of infection (Figure 3). The mean lesion sizes at different time points of infection in control group were not significantly different from niosomes without ALM group ($p > 0.05$). Thus the presence of ALM as an antigen is a critical factor for stimulating the immune response. In ALM and niosomes containing ALM groups, the lesions were developed after 4 and 7 weeks, respectively. The mean lesion size in niosomes containing ALM group was significantly different from control, ALM and niosomes without ALM groups ($p < 0.05$). Although these results exhibited that the niosomes containing ALM can successfully delay the development of lesions and decrease the lesions size in contrast to ALM, unfortunately, this formulation cannot completely prevent cutaneous leishmaniasis. Such a delay in the development of lesions may be due to these fact that the niosomes can prevent the rapid degradation of antigens and act as a slow releasing source of antigens to immune cells (Nakanishi et al., 1997). Furthermore, antigen-presenting cells have more tendency to phagocyte the positively charged vesicles and present the incorporated antigens to other immune cells (Nakanishi et al., 1997). These effects might be very important in the prevention and delay the development of lesions produced by *L. major*. It seems that using the new antigens, improving the niosomal formulations and determining the immune response type (by measuring different interleukins), in future, it will be possible to design the new immunological adjuvant for better prevention of cutaneous leishmaniasis.

Conclusions

ALM was successfully entrapped within the lipid bilayers of the vesicles with high efficiency. Sorbitan ester niosomes had higher EE% in comparison to polysorbate niosomes. By adding Tween to Span formulation, the EE% of ALM was partially decreased in some formulations but stability was increased. The obtained data provide direct evidence that the combination of electrostatic repulsion and steric stabilization can improve the stability of niosomes. In contrast to ALM, Span 40/Tween 40/Chol/CTAB containing ALM has a moderate effect in the prevention of cutaneous leishmaniasis and can successfully delay the development of lesions in BALB/c mice.

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Declaration of interest

The authors alone are responsible for the content and writing of this study.

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