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Preparation of Nanoliposomes by Size Exclusion Chromatography to Entrap Soluble Antigens from *Leishmania Donovani*

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Abstract

In this study, we investigated the ability of nanoliposomes preparation, as a nanoadjuvant, to entrap soluble *Leismania donovani* antigens (SLAs) and release *in vitro*. The parasite reactivation was carried out when inoculated into Rosewell park memorial institute media (RPMI) and incubated at 23 °C for 4 days. *L. donovani* promastigote inoculum (10^4 cell / ml) of 4 days was used to inoculate modified medium of Saline - Neopeptone and Blood agar 9 (SNB 9) to produce promastigote mass. SLAs were extracted from the promastigotes ghost membrane after fourth passages of subculturing in SNB. The membrane pellet obtained was suspended in 5 mM Tris buffer (pH 7.6) and sonicated three times at 4 °C and entrapped in freshly prepared nanoliposomes. Lipids mixture of 4mM Phosphatidylcholine, 2.2 mM Cholesterol and 0.55 mM Phosphatidylethanolamine in a ratio of 7:2:1 were used to prepare nanoliposome. Physio-chemical characterizations of prepared nanoliposomes was performed by using Scanning Electron Microscope (SEM) , Atomic Force Microscope (AFM) and Zeta Potential assays to determine the size, morphology and charge. The efficiency of freshly prepared nanoliposoms to entrap SLAs was determined by measuring the nanoliposome efficiency entrapment (EE). The percentage of EE was 50 and 27.5 of SLAs entrapped nanoliposomes prepared by Sephadex G25 and Sephadex G75, respectively. Moreover, stability of SLAs entrapped nanoliposomes was examined at 4 and 37 °C as a storage temperature.

Keywords: *L. donovani*, Soluble antigens, Nanoliposomes, Efficiency entrapment.

تحضير الجسيمات الدهنية النانوية باستخدام كروموتوكرافيا الترشيح الحجمي لاقتناص المستضدات الذائبة لطفيلي اللشمانيا دونفاني

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الخلاصة

في هذه الدراسة، قمنا بفحص قدرة الجسيمات الدهنية النانويه المحضره ، باعتبارها مساعدات مناعية نانويه ، لاقتناص مستضدات اللشمانيا دونفاني القابلة للذوبان (SLAs) في المختبر. جرى إعادة تنشيط الطفيلي عند تلقيحة في وسط زرعي خاص يعرف بالـ RPMI وحضنت في 23 م° لمدة 4 أيام. وقد استخدمت اللشمانيا دونفاني امامية السوط بعد 4 ايام من تلقيحها وبواقع 10^4خلية / مل لتلقيح الوسط الزرعي

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المعدل المكون من ملح – نيوبيتون وأجار الدم 9 (SNB 9) لإنتاج كتلة من امامية السوط. تم استخراج مستضدات اللشمانيا القابلة للذوبان من غشاء اللشمانيا امامية السوط بعد التمريره الرابعة من المزرعة في الوسط SNB 9. علق راسب الاغشية التي تم الحصول عليها في 5 ملي مولر دارئ تريس (درجة الحموضة 7.6) وتم تكسير الخلايا ثلاث مرات في درجة حرارة 4 م˚ ، ومن ثم اقتناص الجسيمات الدهنية النانوية المحضره بشكل طازج.استخدم خليط الدهون المكون من : 4 ملي مولر من Phosphatidylcholine ، 2.2 ملي مولر من Cholesterol و 0.55 ملي مولر من Phosphatidylethanolamine بنسب 7:2:1 استخدمت لتحضير الجسيمات الدهنيه النانويه. تم تحديد المواصفات الفيزيائية الكيميائية للجسيمات الدهنية النانوية باستخدام مجهر المسح الإلكتروني (SEM)، مجهر القوة الذري (AFM) وقياس زيتا المكافئ ،وذلك لتحديد حجم وشك والمجموعة الكيميائية النشطة والشحنة لهذة الجسيمات. تم تحديد كفاءة الجسيمات الدهنية النانوية الطازجةلاقتناص مستضدات اللشمانيا القابلة للذوبان عن طريق قياس كفاءة اقتناص الجسيمات الدهنية النانوية (EE). بلغت نسبة كفائة الاقتناص 50 و 27.5 من مستضدات اللشمانيا القابلة للذوبان المقتنصة بالجسيمات الدهنية النانوية والمحضره باستخدام سيفادكس G25 و G75 ، على التوالي . علاوة على ذلك، تم فحص استقرار مستوى اقتناص الجسيمات الدهنية النانوية في درجة تخزين 4 و 37 م˚ .

Introduction:

Nanoliposomes, or nanometric versions of liposomes, are colloidal structures formed by the input of energy to a right combination of constituent molecules (mainly phospholipids) in an aqueous solution [1]. Nanoliposomes formulations could be consist of either natural or synthetic lipids. Biodegradable and biocompatible phospholipids and sphingolipids are the lipids that are commonly used to prepare vesicles. Phosphatidylcholines are the widely used lipids due to their appropriate stability and their ability to act against changes in pH or salt concentrations [2]. The selection of the appropriate lipid composition, concentration of bilayers and aqueous phase ingredients such as buffers lead to long term storage. Charge inducing lipid such as phosphotidyl glycerol can be incorporated into liposome bilayers to decrease fusion while cholesterol and sphingomyellin can be included in the formulation to decrease permeability and leakage of encapsulated components [3-5]. The bioavailability of encapsulated components is higher in nanoliposomes than in conventional liposomes due to the better distributions in the organisms, small nanoliposomes with diameters of about 100 nm are frequently used as a delivery system for drugs [6-8].

Preparation of complex nanoliposomes is very challenging by traditional methods, due to the difficulties in the implementation of small particle size and high entrapment efficiency [9]. The most commonly methods for liposomes preparation based on the replacement of an organic solvent by an aqueous media [10]. Tong and his collages [11] investigated the effect of Sephadex G75, G50 and G25 on preparation of nanoliposomes loaded with ginkgolide, and the result indicated that aperture of gels were narrower, particle size of nano-liposomes was smaller (207.13 ~ 89.16 nm).

L. donovani parasite has a digenetic life cycle and exist in two distinct morphologies, the promastigote in sand fly vector ,and the a mastigote in mammalian host [3]. The parasite caused the most severe form of visceral leishmaniasis (VL) (kala-azar or black fever) , a lethal disease if un treated . The disease is endemic in 88 countries on five continents and more than 90% of cutaneous *Leishmania* cases occur in Iran, Afghanistan, Syria, Saudi Arabia, Brazil, and Peru [12]. The *Leishmania* promastigotes are covered with dense surface glycocalyx that is composed of several molecules attached by anchor like surface protease glycoprotein (gp63), glycosylinositol phospholipids (GIPls) and lipophosphoglycan (LPG). All these molecules appear to play certain roles in *Leishmania* infection of macrophages [13]. Numerous attempts have been done to develop an effective vaccine against Leishmaniasis. However, Based on the clinical trials results, none of these vaccines confirmed for widespread vaccination yet, mainly due to the lack of a suitable adjuvant. Using of cationic nanoliposome as a vaccine delivery system is highly regarded [14].

The natural components and biodegradable of nanoliposomes represent an exciting approach to use these particles as nanoadjuvants . The aims of this study is to prepare nanoliposomes by using Sephadex G25 and G75. The characterization of nanoliposomes by AFM, SEM and Zeta potential assays was performed .Extraction and entrapment of soluble antigens from *L. donovani* (SLAs) in nanoliposmes had been investigated . In order to control SLAs releasing from nanoliposomes, the

entrapment efficiency (EE) of nanoliposome was determined *in vitro*. Moreover, the stability of nanoliposomes entrapped SLAs was examined under different storage temperature.

Materials and Methods

Parasite and chemicals

L. donovani parasite was loaned from Biotechnology research center , Al-Nahrain University, Baghdad, Iraq. Sephadex G25 and G75 was purchesed from Pharmacia (Sweden) and Sigma (USA)companies. Cholesterol, Phosphatidylcholine and Phosphatydalethanolamine were obtained from BDH (England). All other chemicals were of the highest purity commercially available.

Parasites reactivate and subculture

Promastigotes of *L. donovani*, was reactivated at 23°C in RPMI for four days and subcultured in the same medium at an average density of 10^4 cell/ml and examined by light microscope. For parasite reproduction, a modified Saline - Neopeptone and Blood agar 9 (SNB9) biphasic medium was used [4].

Parasite harvesting and antigens preparation

Stationary-phase of promastigotes, harvested after the third or fourth passage in liquid culture. The culture centrifuged at 5000rpm for 10 minutes, then the precipitated cells was re- suspend and washed three times with sterile PBS and resuspended at a concentration of 1 g of cell pellet in 50 ml of cold 5 mM Tris-HCl buffer (pH 7.6). The suspension was vortexed six times for 2 min each, with 10 minutes interval of cooling on ice between each vortex. Parasite suspension was then centrifuged at 5000 rpm for 10 minutes. The crude ghost membrane pellet obtained was suspended in 5 ml of 5 mM Tris buffer (pH 7.6) and sonicated three times for 1 min each at 4°C in an ultrasonicator. Finally, centrifuged for 30 minutes at 5000 rpm. The supernatant containing antigens were harvested and stored at -70 °C until used, and protein content in the supernatant was measured by Bradford method [15, 16].

Nanoliposome preparation by Sephadex G 25 and Sephadex G75

Pre-treatment of Sephadex (swelling of dextran gel)

The dry dextran gel would be swollen in water in advance. 6.5 g Sephadex G75 and 20 g Sephadex G25 were added into 50 mL distilled water respectively. Sephadex G75 would be boiled for 3 hours, and the boiling time for G25 would be 1 hour. After the supernatant and fine particles had been removed, the treated gels were made ready for the next step [16].

Nanoliposomes preparation

Nanoliposome was prepared by the method of ether injection. All lipids (4mM Phosphatidylcholine (PC), 2.2 mM Cholesterol (C) and 0.55mM Phosphatidylethanolamine (PEA) were dissolved in 20 mL of ether in the ratio of 7: 2: 1 respectively. Then the organic phase was obtained by adding in 5ml methanol solution of 2mg/ml *leishmania* antigens. The treated gels (5ml) and 0.3 ml of Tween - 80 were added in 30ml of water, as aqueous phase, and stirred (750 rpm) at 74 ^{o}C. Thereafter, organic phase was injected into the aqueous solution, which was magnetically stirred and maintained at 74 ^{o}C to evaporate the ether. When the liquid had been evaporated to 10 mL, 20 mL ice water was added in, continuously stirred for 10 minutes at the ambient temperature. In order to extract nano-liposomes from dextran gels, suspension was passed through a 0.45 μm micron filter [16].

Nanoliposomes characterization

Scanning Electron Microscopy (SEM)

The diameter and morphology of nanoliposome was examined by scanning electron microscopy (SEM) (Inspect S50, Holand) found in the university of Technology , Baghdad, Iraq . Sample was dispersed on glass slide and silver paste used as filament. Then viewed using an accelerating voltage of 15 kilovolt at different magnifications.

Atomic Force Microscopy (AFM)

The shape and morphology of empty liposomal formulations were observed using a NanoWizard®II atomic force microscope (AFM, JPK Instruments, Germany) found in the Chemicals and Petrochemicals research center, Ministry of Industry and Minerals, Baghdad, Iraq. Before AFM imaging, nanoformulations were diluted with distilled water and one drop of diluted dispersion was mounted on the glass slides, air-dried and scanned by the AFM. AFM was operated at room temperature with image resolution of 512 pixels × 512 pixels at a scan speed from 0.9 to 1.2 Hz in air [6].

Zeta potential determination

Zeta potentials of liposomal formulations were determined by dynamic light scattering measurements using Zetaplus analyzer. The analysis was performed at 25°C and after the dispersion was diluted to an appropriate volume with deionized water. The measurements were conducted in triplicate [7].

Liposomal entrapment of *Leishmania* antigens

Standard solution of antigens (0.5mg /ml) was prepared in methanol and then evaporated to form a thin layer film. Two ml of Empty liposomes (ELs) was added, and the mixtures were sonicated for 10min at 60 °C, using a high-energy bath-type sonicator. The liposomal suspensions were allowed to stand at room temperature for 30 minutes. Unentrapped antigens were separated by centrifugation at 13000 rpm for 10 minutes [7].

Entrapment Efficiency (EE) determination

For determination of Ag entrapment efficiency, small aliquots of liposomes (50 µl) were diluted in 950 µl methanol, they were subjected to sonication until liposomes disruption and analyzed for Ag content by HPLC. The %EE was calculated from the amount of incorporated Ag divided by the total amount used at the beginning of preparation multiplied by 100 or as following [8]:

%EE = (amount of incorporated Ag / Total of Ag at beginning) × 100

Stability of nanoliposomes entrepped *L. donovani* antigens

The stability was assessed by comparing different changes in entrapment efficiencies (EE%) of freshly prepared and stored of the complex from light at 4 and 37 °C in sealed conditions at fixed time intervals (0, 3, 6, 9, 12 days) respectively [8].

Results and discussion:

Characterization of nanoliposomes

Scanning Electron Microscope (SEM)

The population of particles about 210 nm in diameter was observed in samples of nanoliposomes prepared by Sephadex G25 Figure-1a, whereas particles with 339 nm in diameter was obtained from Sephadex G75 as present in Figure-1b. The particle size was shown to play a significant role on vesicle trafficking and particles uptake. Large particles of nanoliposomes (0.5–2 µm) are usually taken up by dendritic cells (DC) at the site of injection [9, 10].

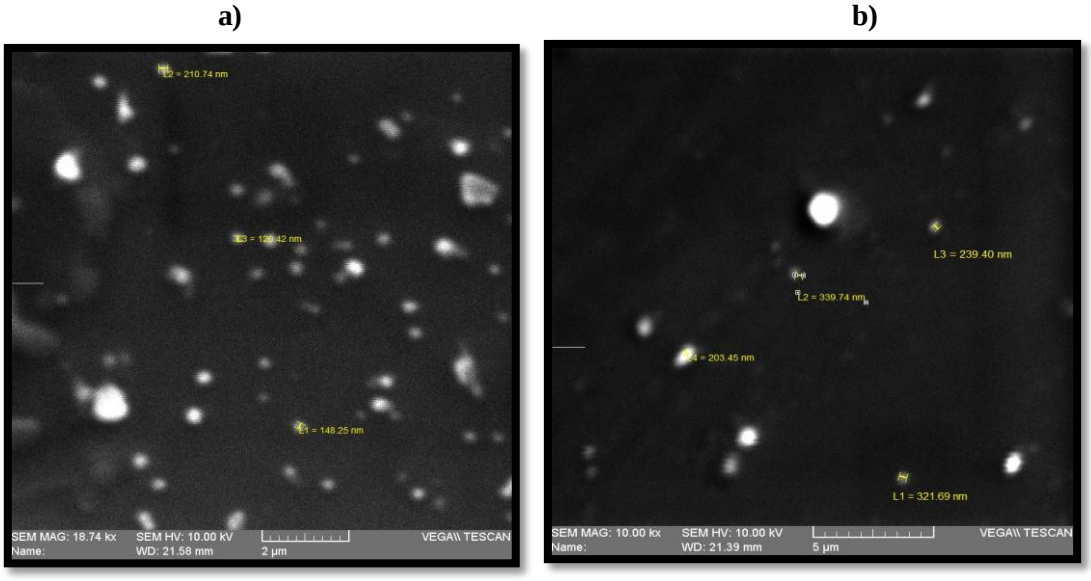


Figure 1- SEM image of nanoliposome , a)prepared by Sephadex G-25 , and b) prepared by Sephadex G-75.

AFM measurement

The images of nanoliposomes from AFM display distinct patterns of adhered nanoliposomes: aggregates and single liposomes as shown with vesicles obtained by Sephadex preparation methods Figure-2.The high resolution of the observed liposome images allows the visualization of the aggregation of the vesicles forming liposomes which were shown to have their origin in the liposome

formation process .Since the observed liposomes are aggregated vesicles, this aggregated structure has a substantial effects on stability, than the single vesicle structure , and consequently effect on the maintaining , shape and function of liposomes. The AFM measurement mode was evaluated the nanoliposome complex in addition to SEM. More steps in future study, for vaccine preparation against leishmaniasis , both SEM and AFM was used to evaluate nanoliposomes complexes.

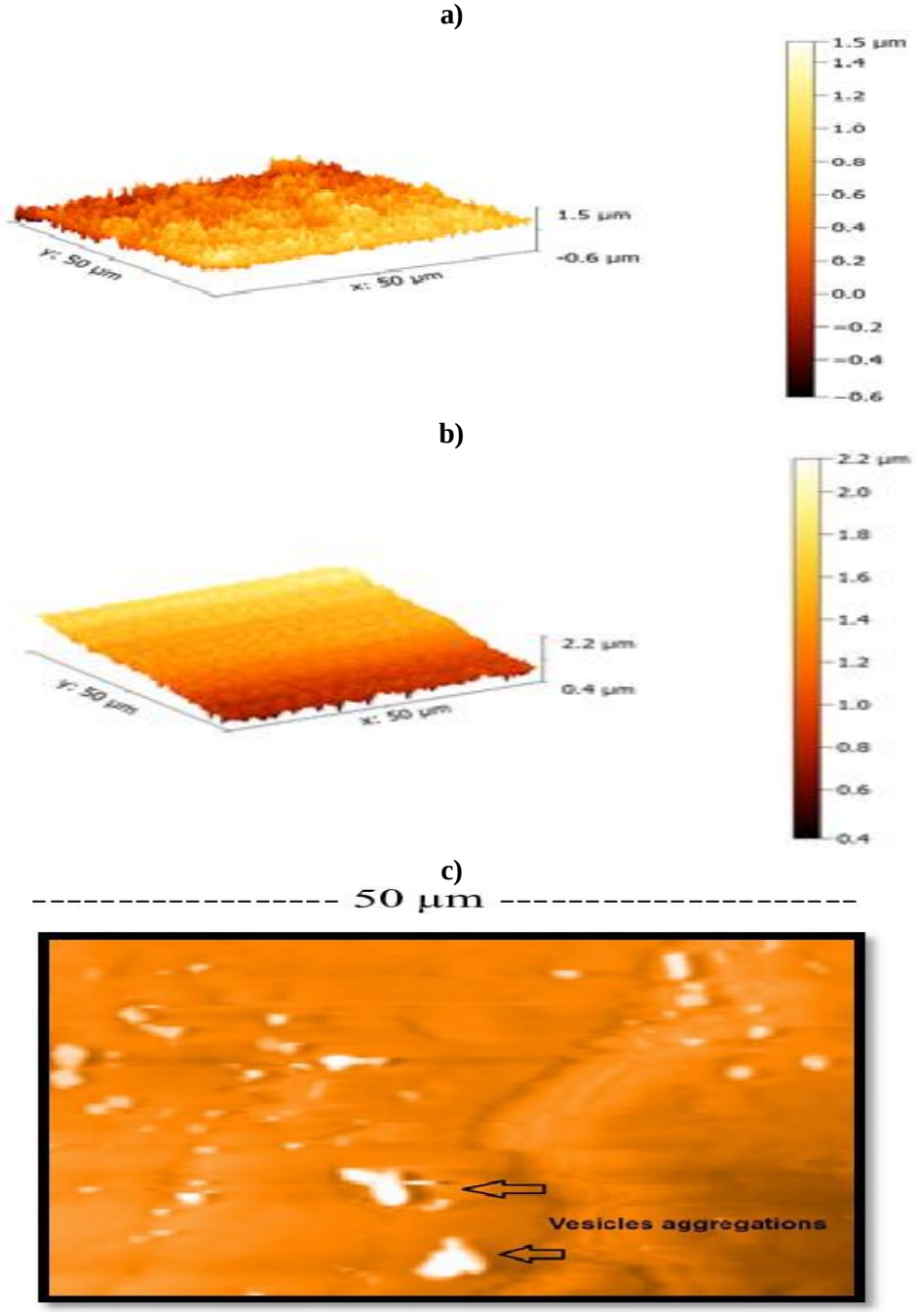


Figure 2- A three-dimensional atomic-force microscopy image of nanoliposomes prepared by **a)** Sephadex G75, **b)** Sephadex G75 method and **c)** Aggregation of nanoliposomes prepared by Sephadex G57 on glass.

The AFM ability to explore samples under variety of environmental conditions, including biological specimens in an aqueous environment or in air at room temperature, makes it a very versatile characterization technique. Unlike the electron microscopical methods, that often require sophisticated sample preparation procedures, the sample preparation for AFM is easy and fast and it allows the material to be preserved in its native state [11, 17]. AFM is being widely used to study the size and adsorption behavior of vesicles on solid substrates since the high resolution can follow the

rupture event of any single vesicle in the lateral direction and enable the quantification of the adsorbed film thickness in the vertical direction [12].

Zeta potential

The zeta potential of SLAs entrapped nanoliposomes prepared by sephadex G25, sephadex G75 methods were (-23.36) and (- 38.86) respectively. It can be seen that the nanoliposomesis is at stable form when the zeta potential value is in range of -16 to - 30 , whereas stability decreased within the range of 30- to – 40. Generally, particles with zeta potentials between -30 mV and +30 mV will have a tendency to aggregate over time [13].

Evaluation of the zeta potential of a nanoliposomepreparation can help to predict of any modification of the nanoliposome surface, controlling the aggregation, fusion and precipitation of nanoliposomes, which are important factors affecting the stability of nanoliposomal formulations [14]. A higher level of zeta potential results in greater electrostatic repulsion forces between the particles. This repulsion leads to greater separation distances between particles in the suspension, reducing aggregation/flocculation caused by Van der Waals interactions .The positive charge on the surface of the resulting particle strongly improves the uptake of both liposomes and the entrapped antigen by macrophages and dendritic cells and their subsequent presentation to responder cells [17 - 20]. Although, the opposite charge between liposome surface and antigen improves the uptake process of the complex, we think that the similar charge did not prevent the uptake of antigen by nanoliposomes. It will facilitate more the releasing of antigens in immune system or during digestion and presenting of released antigens from the complex and induce immune response better than when it presented together.

Efficiency Entrapment (EE) of SLAs

In this study, liposomes lipids and SLAs mixed in a ratio of 43.2 . The EE of the nanoliposomes to entrap SLAs was determined immediately after sonication of vesicles . It can be readily noticed from Table-1, that EE of nanoliposomes prepared by SephadexG25 and Sephadex G75 methods were 50 and 27.5 %, respectively

Table 1- Entrapment Efficiency of nanoliposomes prepared by Sephadex G 25 and Sephadex G 75 methods.

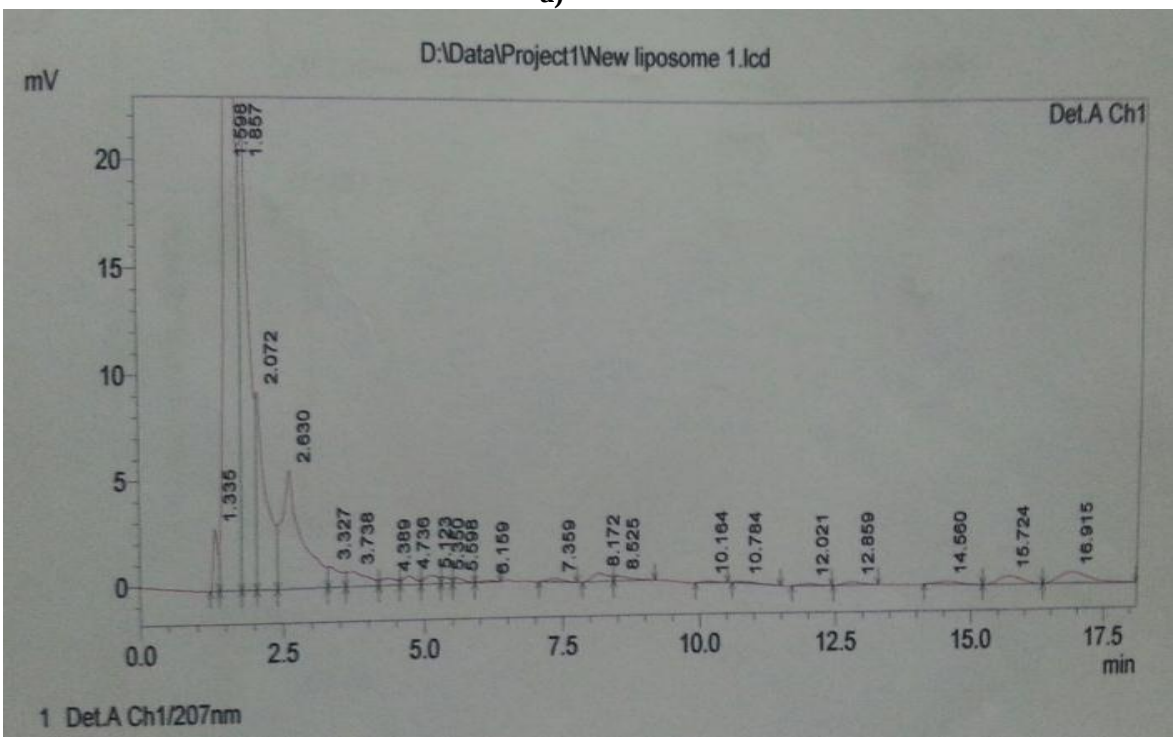
Nanoliposome preparation method	*Leishmania* antigens mg/ml nanoliposome	Un-entrapped antigens (mg/ml)	Absorbance of un-entrapped antigens	Incorporated antigens (mg/ml)	Entrapment Efficiency (% EE)
Exclusion by Sephadex G25	0.4	0.2	0.14	0.2	50
Exclusion by Sephadex G75	0.4	0.29	0.16	0.11	27.5

Numerous attempts have been done to develop a suitable and effective adjuvant to be used in vaccine against Leishmaniasis. The first generation of *Leishmania* vaccine, Badeei et al [14] used SLA entrapped cationic liposome, in the range of 200-400 nm, as a delivery system in vaccine preparation. He was concluded that the EE of SLA in liposomes was 27% in extrusion method (similar to the EE of nanoliposoemes prepared by Sephadex G75 used in this study) and 62% in sonication method. The use of surfactant could have helped the encapsulation process as mentioned in [21]. The EE of liposomes markedly increased from approximately 22% to 65% by increasing lipid /drug ratio from 15 to 30 [22]. High lipid doses may raise concerns of toxicity, reduce the economic feasibility of pharmaceutical scale production and worsen the physical characteristics of the dosage form. The bigger size and solvent used to dissolve the drug have an effect on encapsulation efficiency of nanoparticles, while the solvent have no effect on the release rate [7]. The influence of different formulation variables such as loading methodology, type of main lipid, addition of PEGylated lipid and cholesterol percentage was evaluated to achieve required entrapment efficiency, in vitro release behavior and stability [6].

Stability of nanoliposomes entrapped SLAs

It is worth noting that nanoliposomes showed decreasing in EE of antigens release rate with time under storage temperature of 4 and 37 °C when compared to liposomes prepared at zero time. The EE determination depends on measuring the area of unknown protein in crud soluble antigens at retention time of 5.2 Figure-3. Nanoliposmes stability at 4 °C Figure-4a showed decreasing in EE to 27 and 16 %, while stability at 37 °C Figure-4b revealed decreasing in EE to 8 and 5 % within 12 day of storage of nanoliposomes complexes that prepared by sephadex G25 and sephadex G75 methods, respectively.

a)



b)

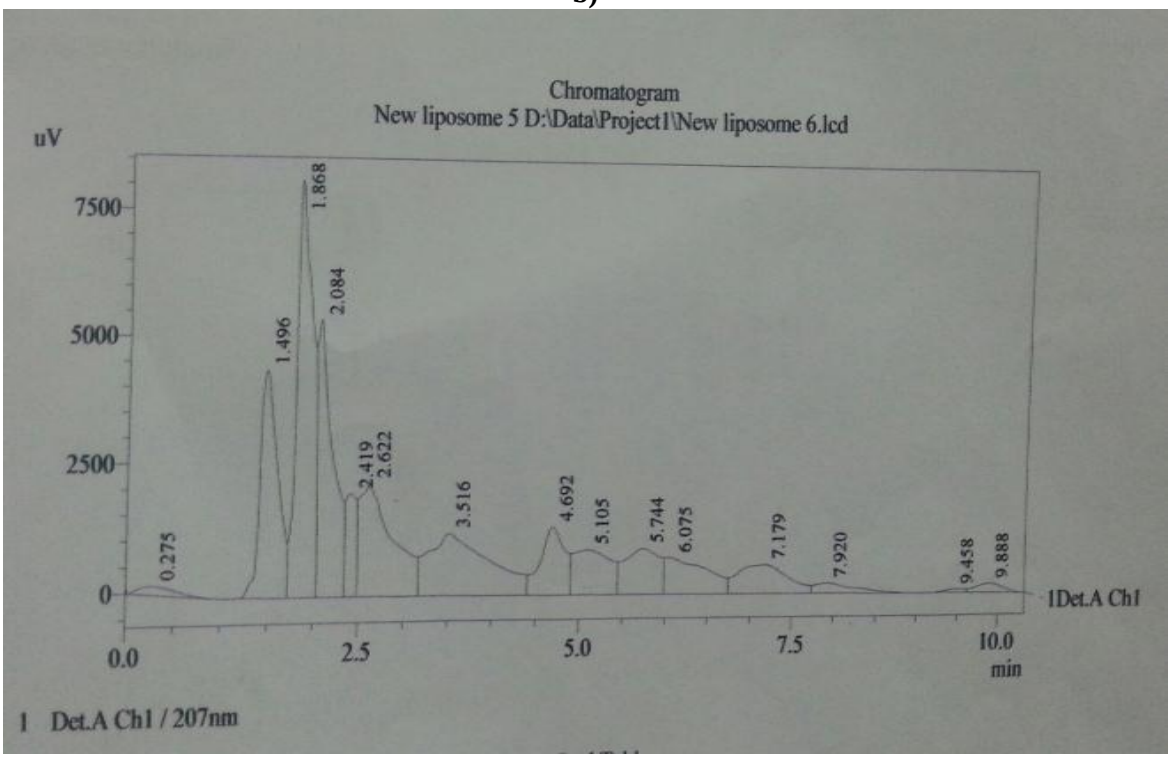


Figure 3- Detection of nanoliposome stability by HPLC prepared by a) Sephadex G25 and b) Sephadex G75 after 10 days of incubation at 4 °C .

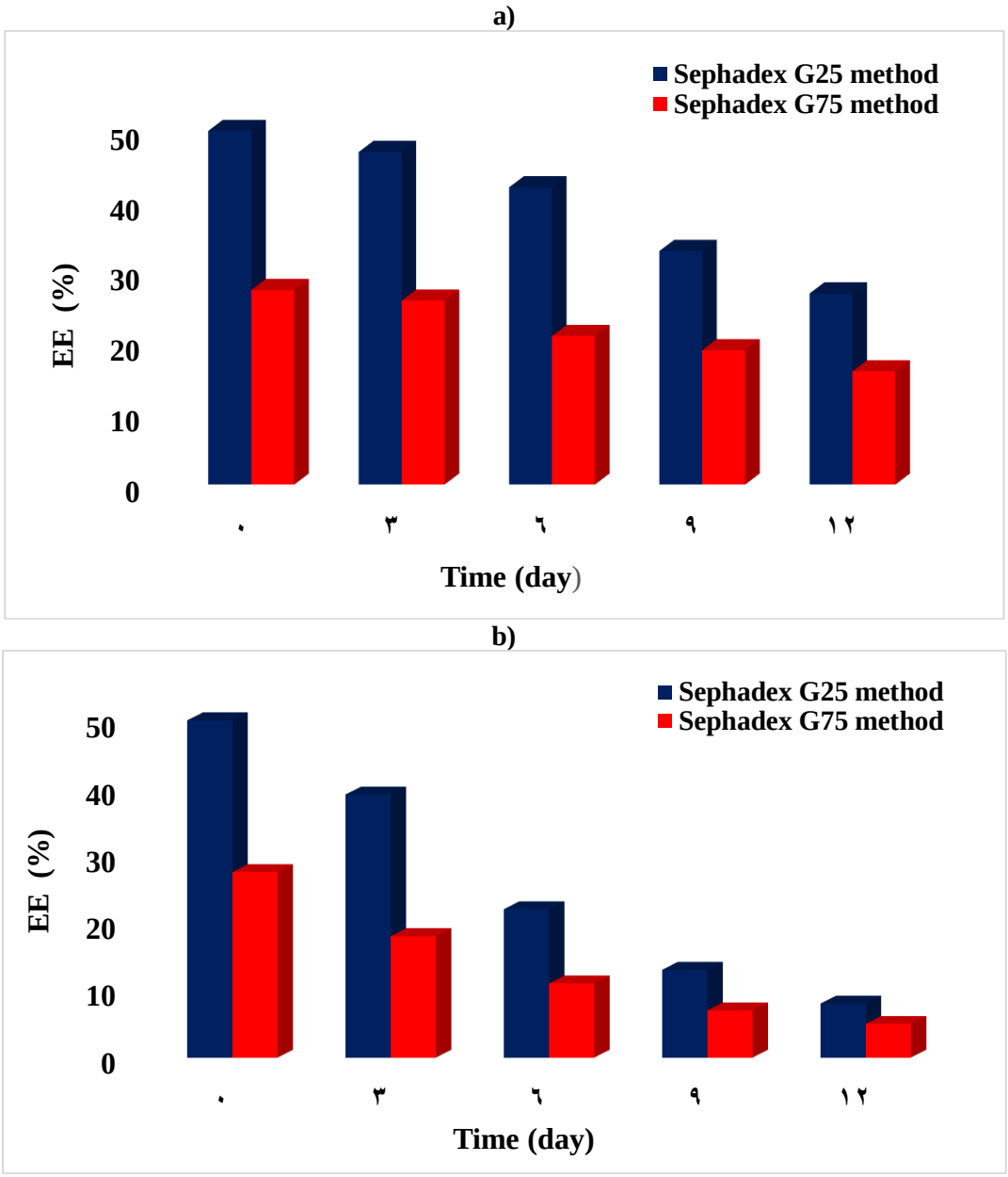


Figure 4- Stability of SLAs entrapped nanoliposomes at (a) 4 °C and (b) at 37 °C analyzed by HPLC.

When the temperature elevated to 37 °C, membrane permeability rate increased, displaying the increase in percentage of SLAs released. This is in agreement with other published findings which have also shown temperature sensitive liposomes was highly unstable under physiological conditions [20]. Liposome entrapment has been shown to stabilize the encapsulated materials against a range of environmental and chemical changes, including enzymatic and chemical modification, as well as buffering against extreme pH and temperature [1].

The degradation percent of nanoliposomes complexes are shown in table (2) when incubated at 4 and 37 °C. Nanoliposomes complexes prepared by sephadex G 25 (46%) and sephadex G75 (41.8%) at 4 °C during 12 day of incubation under dark .Generally, degradation percent were higher at storage temperature of 37 °C than 4 °C .The increment of degradation percent after 12 day was possibly due to the partial aggregation brought by the minimization of high surface-to-volume ratios of liposomes or damage in nanoliposomes structure.

Table 2- Degradation percentage of nanoliposomes at 4 and 37 °C within 12 day

	Stability at 4 ° C			Stability at 37 °C		
Nanoliposomes preparation method	%EE at zero time	%EE at 12 day	degradation (%)	%EE at zero time	%EE at 12 day	degradation (%)
Exclusion by Sephadex G25	50	27	46	50	8	84
Exclusion by Sephadex G75	27.5	16	41.8	28	5	82.1

The stability of fatty acids and vitamin C (FAs-vit C) entrapped in liposomes at 4 ℃ for 3 months presented good stability with relative bigger changes in particle size from (86.8 ± 1.01) nm to (172.6 ± 5.51) nm [5]. The formation and stability of the liposomes are highly dependent on the phospholipid to Cholesterol ratio and it has great impact on the in vitro and *in-vivo* behavior of the carrier. Cholesterol is a common component of liposomes, controlling membrane permeability, providing rigidity to the membrane, stabilizing the bilayer structure and improving plasma stability [23]. Cholesterol addition increases the in vivo stability of the vesicles, resulting in a more rigid structure which further promotes stimulation of $CD8^+$ T-cell responses. Another rational for using cholesterol was to enhance cytoplasmic release of the antigens and avoid the vesicle lysosomal degradation [24, 25]. Although the cholesterol appeared to play a role in the in vivo stability of liposomes, we found that the in vitro stability of nanoliposmes entrapped SLAs revealed variable stability at 4 and 37 ℃ despite the constant concentration of cholesterol used in both preparing methods.

Conclusion:

Different nanoliposomes size can be prepared according to the preparation methods. The entrapment efficiency (EE) of SLAs in all prepared nanoliposomes was achieved with variable percentages. Nanoliposomes entrapped SLAs are more stable at storage temperature of 4˚C than 37˚C within 12 day.

Reference:

1. Mozafari, M., Johanson, C., Hatziantoniou, S. and Demetzos, C. **2008**. Nanoliposomes and their applications in food nanotechnology. *J. Liposome Res*., 18(2), pp:309–327.
2. Batista, C., M., Carvalho, C., M., B. and Magalhaes, N., S., S. **2007**. Lipossomas e suas aplicacoes terapeuticas: Estado da arte. Brazilian *J. Pharmaceut. Sci.*, 43(2), pp:167-179.
3. Chen**,** C. M. and Alli, D. **1987**. Use of fluidized bed in proliposome manufacturing *J. Pharm. Sci.*, 76(5), p:419.
4. Grekov, I., Svobodova, M., Nohynkova, E. and Lipoldova, M. **2011** .Preparation of highly infective Leishmaniapromastigotes by cultivation on SNB-9 biphasic medium. *Journal of Microbiological Methods*, 87, pp: 273–277.
5. Yang**,** S., Liu, C., Liu, W., Yu, H., Zheng, H., Zhou, W. and Hu, Y. **2013**. Preparation and characterization of nanoliposomes entrapping medium-chain fatty acids and vitamin C by lyophilization. *Int. J. Mol. Sci*., 14, pp: 19763-19773.
6. Mozafari, M., Reed, C., Rostron, C. and Hasirci, V. **2005a**. Construction of a gene delivery vector using anionic liposomes. In: Mozafari, M. R., Mortazavi, S.M. (Eds.), *Nanoliposomes: From Fundamentals to Recent Developments*. Trafford Publishing Ltd., Oxford, UK, pp:101–111.
7. Mozafari**,** M., Reed, C., Rostron, C. and Hasirci, V. **2005b**. A review of scanning probe microscopy investigations of liposome–DNA complexes. *J. Liposome Res*., 15, pp: 93–107.
8. Darani**,**K. and Mozafari ,M.R. **2010**. National nutrition and food technology research institute. *Int. J. Nanosci. Nanotechnol*., 6(1), pp: 3-13.
9. Huang, Q., Hailong, Y. and Qiaomei, R. **2010**. Bioavailability and delivary of nutraceuticals using nanotechnology. *J .Food Science*, 75(1), pp:50-57.

10. Wagner, A. and Vorauer-Uhl, K. **2011**. Liposome technology for industrial purposes. *J. Drug Delivery (*Article ID 591325), pp:1-9.
11. Tong, Y., Chen, Y., Pan, J., Huang, L. and Wang, R. **2010**. Effect of Dextran Gel on preparation of nano-liposomes loaded with Ginkgolide. *Bull. Korean Chem. Soc.*, 31(9), pp:2542-2546.
12. Singh, S. **2006**. New developments in diagnosis of leishmaniasis , *Indian Med Res.*, 123, p: 311.
13. Beverley, S.M. and Turco, S.J. **1998**. Lipophosphoglycan (LPG) and the identification of virulence gene in the protozoan parasite Leishmania. *Trends Microbiol.*, 6, pp:35-40.
14. Badiee, A., Jaafari, M., Khamesipour, A., Abbasi, A. and Saberi, Z. **2012**. Preparation and characterization of nanoliposomes containing soluble *Leishmania* antigens and MPL adjuvant. *Res. Pharma. Sci.*, 7(5), p: S238.
15. Bradford, M. **1976**. Arapid and sensitive method for the quantitation of microgram quantities of protein utilizating the principle of protein-dye . *Analytic Biochemistry*, 72, pp:248-254.
16. Mozafari, M. **2005**. Liposomes: an overview of manufacturing techniques, *Cel. Mol. Bio. Let*, 10, pp: 711–719.
17. Sharma, U. and Singh, S. **2009**. Immunobiology of leishmaniasis . *J. Experimental Bio*., 47, pp: 412-423.
18. Mayer**,** L., Cullis, P. and Balley, M. **1998**. *Medical application of liposome*, Elsevier Science BV: New York.
19. Afrin**,** F., Rajesh, R., Anam, K., Gopinath, M., Pal, S. and Ali, N. **2002**. Characterization of *Leishmania donovani* Antigens Encapsulated in Liposomes That Induce Protective Immunity in BALB/c Mice. *Infection and Immunty*, 70(12), pp: 6697–6706.
20. Haeri, A., Alinaghian, B., Daeihamed, M. and DadashzadehS. **2014**. Preparation and Characterization of Stable Nanoliposomal Formulation of Fluoxetine as a Potential Adjuvant Therapy for Drug-Resistant Tumors . *Iranian Journal of Pharm. Res.*, 13 (supplement), pp: 3-14.
21. Derakhshandeh,K., Soheili,M ., Dadashzadeh,S.and Saghiri,R. **2010**. Preparation and invitro characterization of 9- nitrocamptothecin – loaded long circulating nanoparticles for delivery in cancer patients. *Int. J. Nanomedicine*, 5, pp: 463-471.
22. Mordstrom**,** P. **2011**.Formation of polymeric nanoparticles encapsulating and releasing a new hydrophobic cancer drug. MSc thesis in Materials and Nanotechnology, Department of Chemical and Biological Engineering , Chalmers University of Technology, Goteborg, Sweden.
23. Haeri A, Sadeghian S, Rabbani S, Anvari MS, Boroumand MA and Dadashzadeh S. **2011**. Use of remote film loading methodology to entrap sirolimus into liposomes: preparation, characterization and in vivo efficacy for treatment of restenosis. *Int. J. Pharm*., 414, pp:16-27.
24. Ignatius, R., Mahnke, K., Rivera, M., Hong, K., Isdell, F., Steinman, R.M., Pope, M. and Stamatatos, L. **2000**. Presentation of proteins encapsulated in sterically stabilized liposomes by dendritic cells initiates CD8+ T-cell responses *in vivo*. *Blood*, 96, pp: 3505–3513.
25. Hafez, I.M., Maurer, N. and Cullis P.R. **2001**. On the mechanism whereby cationic lipidspromote intracellular delivery of polynucleic acids. *Gene Therapy*, 8, pp: 1188–1196.