Preparation of Nanoliposomes Incorporated *Leishmania donovani* Antigens
تحضير مستضد اللشمانيا الحشوية المغروزة في الجسيمات الدهنية النانوية

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Abstract

This study was designed to incorporate *leishmania donovani* antigens in nanoliposomes prepared by size exclusion (using Sephadex G25) and organic solvent (using Chloroform). Lipids mixture of 4Mm Phosphatidylcholine, 2.2mM Cholesterol and 0.55mM Phosphatidylethanolamine in a ratio of 7:2:1 was depended in two nanoliposome preparation methods. Physio-chemical characterizations of prepared nanoliposomes was performed by using Scanning Electron Microscope (SEM), Fourier transform infrared spectroscopy (FTIR) and Zeta Potential assays to determine the size, morphology, chemical active group and charge . Parasite reactivation was carried out when inoculated into RPMI and incubated at 23 □ C for 4 days. Soluble *Leishmania* Antigenes (SLAs) were extracted from the promastigotes ghost membrane after fourth passages of subculturing in SNB9. The extracted SLAs were entrapped in prepared. The percentage of nanoliposomes entrapment efficiency (EE) was 62 and 50 of SLAs for chloroform and Sephadex G25 methods, respectively. Moreover, stability of SLAs entrapped nanoliposomes at 4 and 37 □C, as storage temperature, was examined. The stability at 4 °C showed decreasing in EE to 32 and 16 %, while stability at 37 °C revealed decreasing in EE to 16 and 8 % within 12 days of storage for nanoliposomes prepared in both methods, respectively.

Key words: Nanoliposome, soluble leishmania antigen, Sephadex G25, Chloroform

الملخص

في هذه الدراسة، تم استخدام طريقتين لتحضير الجسيمات الدهنية النانوية، الطريقة الاولى اعتمدت حجم الإقصاء و باستخدام السفادكس (G25) والطريقة الثانية هي طريقة استخدام المذيبات العضوية (باستخدام الكلوروفورم). اعتمدت نسبة تحضير من 1:2:7 لمركبات 4 ملي مولار من مركب الفوسفاتيديل كولين و 2.2 ملي مولار من مركب الكولسترول و 0.55 ملي مولار من مركب الفوسفاتيديل ايثانولامين في طريقتي التحضير. تم إجراء التوصيف الفيزيائي الكيميائي للجسيمات الدهنية النانوية باستخدام مجهر المسح الإلكتروني (SEM)، ومطياف فورييه بالأشعة تحت الحمراء (FTIR) وزيتا التكافئية لتحديد حجم وشكل والمجموعة الكيميائية النشطة والشحنة. ونظرا لكون مكونات الجسيمات النانوية الدهنية عبارة عن مركبات طبيعية, كانت فكرة الدراسة في استخدامها كمتممات نانوية تحمل مستضدات طفيلي اللشمانيا الحشوية الذائبة خارج الجسم الحي. تم تنشيط الطفلي على وسط (RPMI) وفي درجة حضن 23 م لمدة 4 ايام ليتم بعدها استخلاص المستضد من غشاء الطفيلي بعد التمرير الرابع من زراعة الطفيلي في وسط (SNB9) ليتم قنصها في الجسيمات المحضرة انيا. تم احتساب كفاءة الاحتفاظ او انحباس المستضدات في الجسيمات المحضرة وكانت 62 و 50 بطريقتي استخدام الكلوروفورم والسيفادكس على التوالي. اضافة الى ذالك, فقد تم احتساب ثباتية الجسيمات القانصة للمستضدات الذائبة في درجتي حرارة تخزين 4 و37 م □ , وظهرت النسبة المئوية لثبات هذا المعقد بقيمة 32 و 16 في درجة حرارة تخزين 4م □ في حين بلغت 16 و 8 في درجة حرارة تخزين 37 م □ للجسيمات المحضرة باستخدام الكلوروفورم والسيفادكس (G25) وخلال 12 يوم وعلى التوالي.

الكلمات الدالة: الجسيمات الدهنية النانوية، طفيلي اللشمانيا الحشوية الذائبة، السفادكس (G25)، كلوروفورم

Introduction

Leishmaniasis is one of the most diverse and complex diseases caused by an obligate intra cellular protozoan parasite belonging to the genus *Leishmania*. The most severe form of the disease, lethal if un treated, is caused by species of *Leishmania donovani*. The parasites have two distinct morphologies, the promastigote in sand fly vector, and the amastigote in mammalian host. The disease affects approximately 12 million people every year in 88 countries on five continents numerous attempts have been done to develop an effective vaccine against Leishmaniasis [1,2].

However, based on the clinical trials results, none of these vaccines were confirmed for wide spread vaccination yet, mainly due to the lack of a suitable adjuvant [3].

Nanoliposomes, or nanometric versions of liposomes, are colloidal structures (mainly phospholipids) in an aqueous solution [4]. The key common characteristic of bilayer-forming molecules is their polar and nonpolar regions (amphiphilicity) [5]. Several liposomes based formulations have been approved for human use or reached advanced clinical development in existing vaccine. Modern, effective vaccines rely on a combination of a purified antigen against which an immune response is desired and an adjuvant that triggers the innate immune system to enhance the magnitude and quality of the generated immune response [6].

In this study, two methods of nanoliposome preparation were used to entrap extracted *L. donovani* antigens. Characterization of nanoliposmes was determined by Scanning Electron Microscope (SEM), Fourier transform infrared spectroscopy (FTIR) and Zeta- potential assays.

Materials and Methods

Parasite and chemicals

Lieshmania donovani parasite was obtained from Biotechnology research center, Al-Nahrain University, Baghdad, Iraq. Sephadex G25 was purchased from Pharmacia (Sweden). Chloroform from BDH (England). Cholesterol, Phosphatidylcholine and Phosphatydalethanolamine were obtained from BDH (England). All other chemicals were of the highest purity commercially available.

Culturing of *L. donovani*

Promastigotes of *L. donovani* was reactivated at 23°C in RPMI for four days and subcultured in the same medium at an average density 10^4 cell/ml and examined by light microscope. For parasite reproduction, a modified Saline - Neopeptone and Blood agar 9 (SNB9) biphasic medium [7] was used.

L. donovani antigens preparation

Stationary-phase of promastigotes, harvested after the third or fourth passage in liquid culture. The culture centrifuged at 5000rpm for 10 min , then the precipitated cells were re- suspend and washed three times with sterile PBS and resuspended at a concentration of 1.0 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 a 10- min interval of cooling on ice between each vortex. Parasites suspension was then centrifuged at 5000 rpm for 10 min. The crude 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 min at 5000 rpm. The supernatant containing antigens were harvested and stored at -70°C until used. The protein content in the supernatant was measured by the procedure described by Bradford (1976) [8,9].

Nanoliposome preparation by Chloroform injection

The lipidmixtureof 4mM Phosphatidylcholine (PC), 2.2mM Cholesterol (C) and 0.55mM Phosphatidylethanolamine (PEA) at a ratio 7:2:1 was dissolved in 20 ml chloroform and dried under reduced pressure in a rotary evaporator (90 rpm) at 65°C to form a thin lipid film. Evaporation was continued for 2 h after the dry residue appeared and complete remove all traces of the solvent. The film was then hydrated with 5ml ammonium sulfate (300 mM) at 65°C for 1 h. The prepared multi vesicular suspensions were extruded five times through each of 200 and 100 nm pore size filter to obtain a narrow size distribution. The extrusion was carried out at 65°C to maintain vesicles above phase transition temperature [10].

Nanoliposome preparation by Sephadex G 25 Pre-treatment of Sephadex (swelling of dextran gel)

The dry dextran gel would be swollen in water in advance. Twenty gram of Sephadex G25 were added into 50 mL distilled water and boiled for 1 hour. After the supernatant and fine particles had been removed, the treated gels were made ready for the next step.

Nanoliposomes preparation

Nanoliposomes were 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 at a ratio 7: 2: 1 respectively. Then the organic phase was obtained by adding 5ml methanol containing 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. Twenty ml of cold water was added to the concentrated solution (10ml) after evaporation. In order to obtain nanoliposomes from dextran gels, suspension was passed through a 0.45 µm filter (9).

Nanoliposomes characterization

Scanning Electron Microscopy (SEM)

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

FTIR measurement

FTIR spectra of nanoliposomes precipitated in KBr disks were recorded on a Tensor 27- Pruker (German) spectrometer. The scanning was done in the range 1000–4,000 cm-1 with speed of 2 mm/s at a resolution of 4 cm-1 at room temperature. The band width was measured at 10% of height of the peaks [11].

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 [12].

Nanoliposomal incorporated with *Leishmania* antigens

Leishmania antigens (0.5mg /ml) was prepared in methanol and then evaporated to form a thin layer film. Following the addition of 2ml of nanoliposomes , 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 min. Incorporated antigens were separated by centrifugation at 13000 rpm for 10 min. [12].

Entrapment Efficiency (EE) determination

For determination of Ag entrapment efficiency, small aliquots of liposomes (50 µl) were diluted in 950 µl methanol, 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: %EE = (amount of incorporated Ag / Total of Ag at beginning) × 100.

Stability of nanoliposomes incorporated Leishmania Antigen

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 according to Yang *et al* (2013) [13].

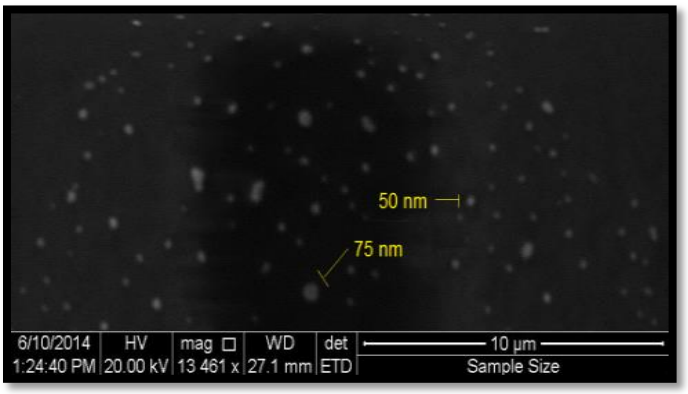
Results and Discussion

Characterization of nanoliposomes

Scanning Electron Microscope (SEM)

Results showed that nanoliposmes prepared by chloroform method revealed vesicles with less than 75nm in diameter Figure (1), while population of particles with not more than 210nm in diameter was observed in samples of nanoliposomes prepared by Sephadex G25 Figure (2) . Large particles of nanoliposomes(0.5–2 µm)are usually taken up by dendritic cells (DC) at the site of injection , whereas small particles (<200 nm) are freely drained into the lymph nodes (LNs) wherein are taken up by phagocytic cells (DCs and macrophages), suggesting that the location of particle uptake is highly dependent on the particle size [14]. Therefore, the particle diameters of nanoliposmes prepared by Sephadex G25 method uptake by DC and drain by LNs. On the other hand, nanoliposomes vesicles around 75nm or less, prepared by chloroform method, are mostly drained into LNs after injection.

A)



B)

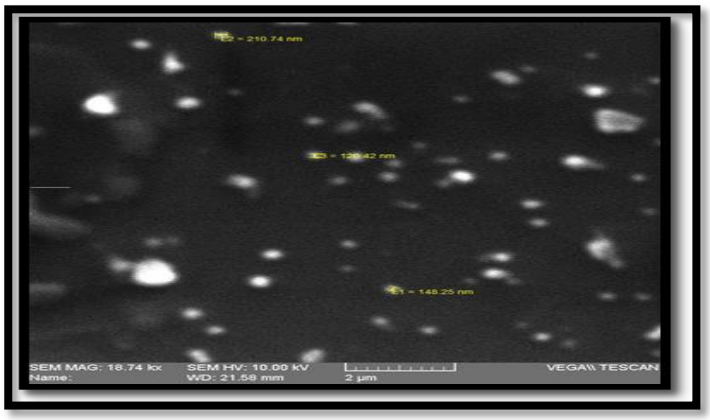


Fig. (1): Scanning electron microscope image of nanoliposomes prepared by A) Chloroform and B) Sephadex G25.

FTIR

Results shown in Figure (2), that the phospholipid band analyzed were observed between 1200 and 970 $cm^{-1.}$ Nzai and Proctor [15] explained that there is a band determined by FTIR referred to the phospholipid appeared between 1200 and 970 cm^{-1} due to both P-O-C and PO2 vibrations. The existence of C-H stretching vibrations of CH2 and CH3 groups due to the addition of cholesterol in nanoliposome preparation. The vibrational frequency of CH2 in 3385 and 2852 cm^{-1} as asymmetric and symmetric stretching. While, the vibration frequency of CH3 was observed at 2921 and 2852 cm^{-1} as asymmetric and symmetric stretching, respectively. Sikkandar *et al* [16] referred to the presence of the Cholesterol that can be characterized by the bands between 2800-3000 cm^{-1} due to C-H stretching vibrations of CH2 and CH3 groups. A phosphate group gives nanoliposomes a negative charge, and this enhance more the release of negative proteins from *Leishmania* antigens entrapped in liposomes after exposure to solvent and sonication.

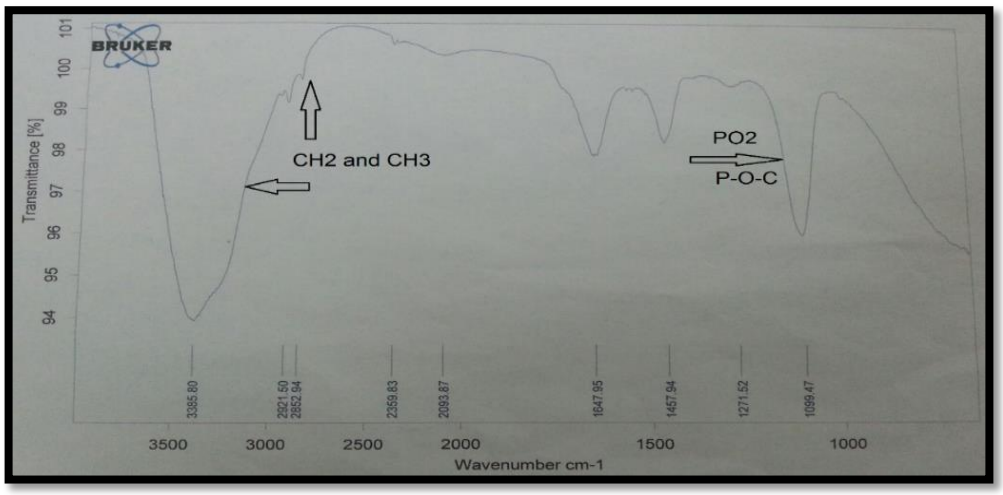


Fig. (2): Overlaid FTIR Spectra of empty nanoliposome prepared by chloroform and Sephadex G25 methods.

Zeta potential

Results showed that the zeta potential of SLAs entrapped nanoliposomes prepared by sephadex G25 and chloroform methods were (- 23.36) and (- 31.94) respectively .It can be seen that stability of nanoliposomesis at beginning of dispersing when the zeta potential value is in range of -16 to -30. While it appeared a moderate stability within the range of (- 30) to (-40) [17]. Sample particles with zeta potentials of between -30 mV and +30 mV will have a tendency to aggregate over time. 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 [18].

Efficiency Entrapment (EE) of SLAs

The ratio of Lipids to Antigens (L/A), as crud soluble antigens, was 43.2. Immediately after formulation of SLAs entrapped nanoliposomes, EE was determined from sonicated vesicles. It can be readily noticed that EE of SLAs nanoliposomes prepared by Chloroform and Sephadex G25 methods were 62 and 50 %, respectively Table (1).

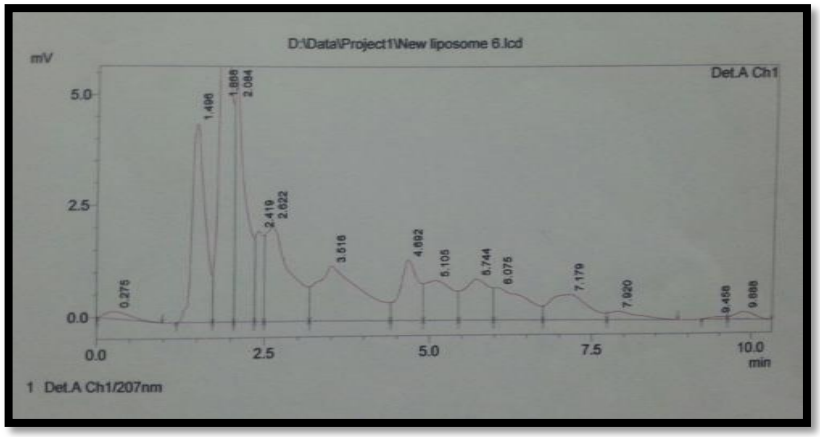
Stability of nanoliposomes incorporated SLAs

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 depend on measuring of the area of unknown protein in crud soluble antigens , at retention time of 5.1, and determined the concentration by the standard curve equation as shown in figure 3 (a and b) . 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 [5].

Table (1): Entrapment Efficiency of nanoliposomes prepared by different methods. Ag concentration determined by Bradford method, and Lipids/Antigens ratio is 43.2 .

Nanoliposome preparation method	*Leishmania* Antigen mg/ml nanoliposome	Un-entrapped Antigen (mg/ml)	Absorbance of un-entrpped Antigen	Incorporated Antigen mg/ml	Entrapment Efficiency (% EE)
Thin film in Chloroform	0.4	0.15	0.136	0.25	62
Exclusion by Sephadex G25	0.4	0.2	0.14	0.2	50

A)



B)

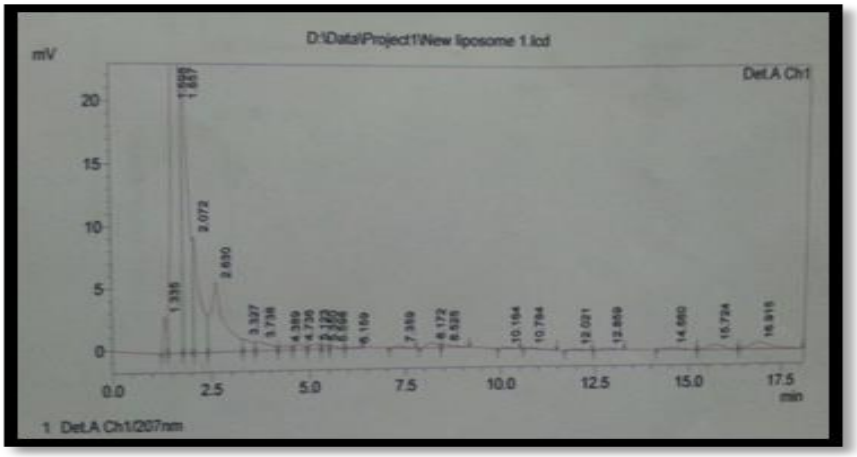


Fig. (3): Detection of nanoliposome stability prepared A) by chloroform and B) by Sephadex G25methods analyzed by HPLC after 10 days of incubation at 40°C . Rt 5.1 is the indicator of antigen protein releasing.

The results of nanoliposmes entrapment stability at 4 °C Figure (4 a) showed decreasing in EE to 32 and 27%, while stability at 37 °C Figure (4 b) revealed decreasing in EE to 16 and 8 % within 12 day of storage of nanoliposomes complexes that prepared by chloroform and sephadex G25, respectively. 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 were highly unstable under physiological conditions [11].

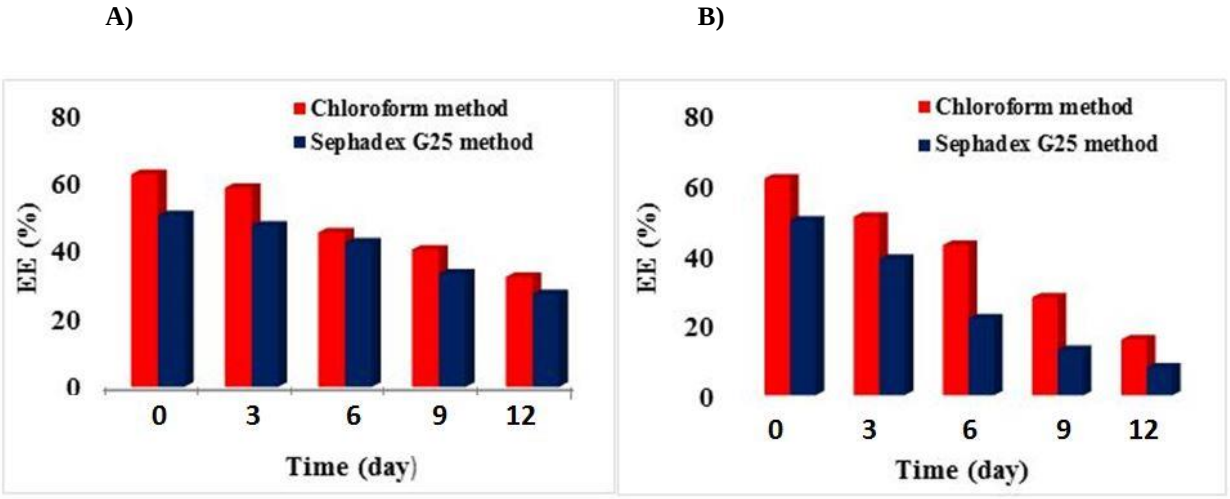


Fig. (4): Stability of SLAs entrapped nanoliposomes (A) at 4^0 C and (B) at 37 °C analyzed by HPLC , after different periods .

The degradation percent of entrapment nanoliposomes complexes are shown in table (2) when incubated at 4 and 37 °C. Nanoliposomes complexes prepared by chloroform method revealed rather high degradation percent (48.3%) when compared with nanoliposomes prepared by sephadex G25 (46%) at 4 °C after 12 day of storage in dark .Generally, degradation percentage were higher at storage temperature of 37°C (74.2 and 84 %) 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.

Methods of Nano-liposomes preparation	Stability at 4 °C			Stability at 37 °C		
	%EE at zero time	%EE at 12 day	Nano-liposomes degradation (%)	%EE at zero time	% EE at 12 day	Nano-liposomes degradation (%)
Thin film in Chloroform	62	32	48.3	62	16	74.2
Exclusion by Sephadex G25	50	27	46	50	8	84

Conclusion

SLAs had been successfully incorporated in nanoliposomes prepared freshly by size exclusion and organic solvents methods. Entrapment Efficiency (EE) and stability of nanoliposomes , incorporated SLAs, depend on type of preparation method, even when the same ratio of nanoliposomes components had been used.

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