

# ***Leishmania donovani* Soluble Antigens (SLAs) Entrapment In Nanoliposomes Prepared By Size Exclusion Chromatography**

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## **Abstract**

The use of biodegradable nanoparticles as vaccine adjuvant with entrapped antigens represents an exciting approach for controlling the release of vaccine antigens and optimizing the desired immune response. Because of the natural components of nanoliposomes, we investigated the ability of prepared nanoliposomes, as a nanoadjuvant, to entrap soluble *Leishmania donovani* antigens (SLAs) in order to be used in a vaccine against Leishmaniasis. In this study, parasite reactivation was carried out when inoculated into 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 (SNB9) to produce promastigote mass. SLAs were extracted from the promastigotes ghost membrane after fourth passages of subculturing in SNB9. The extracted SLAs then entrapped in nanoliposomes prepared freshly. 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 SEM, AFM and Zeta Potential assays to determine the size, morphology, chemical active group and charge. The efficiency of freshly prepared nanoliposomes 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 storage temperature.

## **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 . The phospholipid molecules used in the structure of lipid vesicles are the main component of naturally occurring bilayers. The key common characteristic of bilayer-forming molecules is their polar and nonpolar regions (amphiphilicity)(1).Liposomes can be manufactured at small diameter (<200 nm), permitting terminal sterile filtration and improved lymph node targeting and producing new adjuvants that can direct appropriate immunity. Therefore , vaccine design have shown that even in the context of the same antigen different outcomes are achieved as a function of the co-administered adjuvant . (2) .

Leishmaniasis is one of the most diverse and complex diseases caused by an obligate intra cellular protozoan parasite belonging to the genus *Leishmania*. It manifests mainly in 3 clinical forms; visceral leishmaniasis (VL),cutaneous leishmaniasis (CL) and muco cutaneous leishmaniasis (MCL), of which VL is the most severe form of the disease , lethal if un treated , is caused by species of *Leishmania donovani* complex. The parasites have a digenetic life cycle and exist in two distinct morphologies ,the promastigote in sand fly vector ,and the a mastigote in mammalian host. The disease affects approximately 12 million people every year in 88 countries on five continents (3).In this study , the SLAs from *L. donovani* parasite was extracted and entrapped in freshly prepared nanoliposomes . Sephadex G25 and G75 used to prepare nanoliposomes and characterized by FTIR , AFM, SEM and Zeta potential assay .Entrapment efficiency (EE) of nanoliposome entrapped SLAs was investigated and storage temperature also determined .

## **Materials and Methods**

### **Parasite and chemicals**

*Lieshmania donovani* parasite was loaned from Biotechnology research center , Al-Nahrain university , Baghdad, Iraq. Sephadex G25 and G75 was purchased 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 (4) was used.

### **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 min, then the precipitated cells was 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. Parasite suspension was then centrifuged at 5000 rpm for 10 min. 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 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) (5,6).

### **Nanoliposome preparation by Sephadex G 25 and 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.

### **Nanoliposomes preparation**

Nanoliposome was prepared by the method of ether injection. All lipids (4mM Phosphatidylcholine (PC), 2.2m MCholesterol (C) and 0.55m MP hosphatidylethanolamine (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 °C. Thereafter, organic phase was injected into the aqueous solution, which was magnetically stirred and maintained at 74 °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 min at the ambient temperature. In order to extract nanoliposomes from dextran gels, suspension was passed through a 0.45 µm micron filter (6).

### **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. Following the addition of 2ml of ELs , 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. Unentrapped antigens were separated by centrifugation at 13000 rpm for 10 min (7).

### **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:

$$\%EE = (\text{amount of incorporated Ag} / \text{Total of Ag at beginning}) \times 100.$$

### **Stability of Nanoliposomes entraped *Leishmania* Antigen**

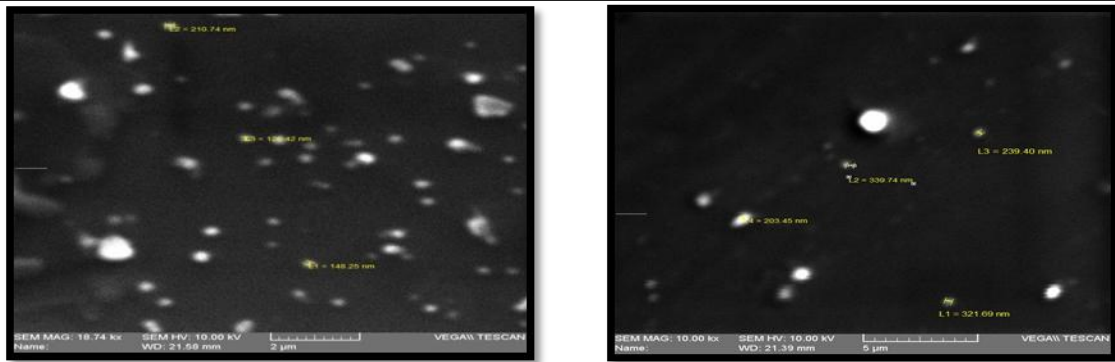
The stability was assessed by comparing different changes in entrapment efficiencies (EE%) of freshly prepared and stored of the complex from light at 4 and 37 °C in sealed conditions at fixed time intervals (0,3,6,9,12 days) respectively (8).

## **Results and discussion**

### **Characterization of nanoliposomes**

#### **Scanning Electron Microscope (SEM)**

In this study , population of particles with not more than 210nm in diameter was observed in samples of nanoliposomes prepared by Sephadex G25 (Figure 1). The particle size of nanoliposomes with not less than 203 nm in diameter was obtained from Sephadex G75 preparation method as present in 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 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 (9).



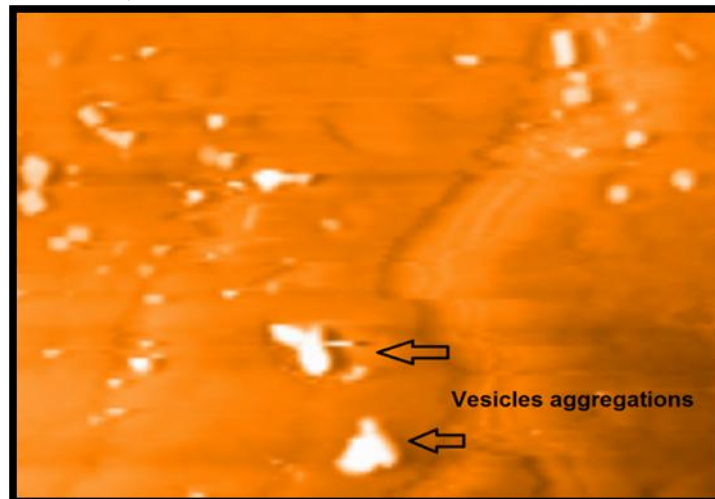
Figure(1):SEM image of nanoliposome

Figure (2) : SEM image of nanoliposome prepared by Sephadex G-75.

### AFM measurement

Atomic force microscopy has enabled direct visualization of the liposome structure supported on glass surface under air in aqueous medium as used in this investigation. The images display distinct patterns of adhered nanoliposomes: aggregates and single liposomes as shown with vesicles obtained by Sephadex preparation methods (Figure 3). 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 on the maintaining , shape and function .

----- 50  $\mu$ m -----



Figure(3):A three-dimensional atomic-force microscopy image of nanoliposomes prepared by Sephadex G75 method on glass .

### Zeta potential

The zeta potential and particle size are the two most important properties that determine the in vivo fate of the nanoliposomes when used as a vaccine .Zeta potential is the overall charge of lipid vesicles acquires in a particular medium. It is a measure of the magnitude of repulsion or attraction between particles in general and lipid vesicles in particular (10) .

In this study , 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 stability of nanoliposomes is at beginning of dispersing when the zeta potential value is in range of 16- to 30- , while it appeared in medium stability within the range of 30- to 40 – (11). Sample particles with measured 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 (12) .

### Efficiency Entrapment (EE) of SLAs

In this study , the ratio of Lipids/Antigens , as crud soluble antigens , is 43.2 .Immediately after formulation of SLAs entrapped nanoliposomes, EE was determined from sonicated vesicles .From table (1) , it can be readily noticed that EE of SLAs in nanoliposomes prepared by SephadexG25 and Sephadex G75 methods were 50 and 27.5 %.

**Table (1):** Entrapment Efficiency of nanoliposomes prepared by Sephadex G 25 and Sephadex G 75 methods .Ag concentration determined by Bradford method, and Lipids/Antigens ratio is 43.2 .

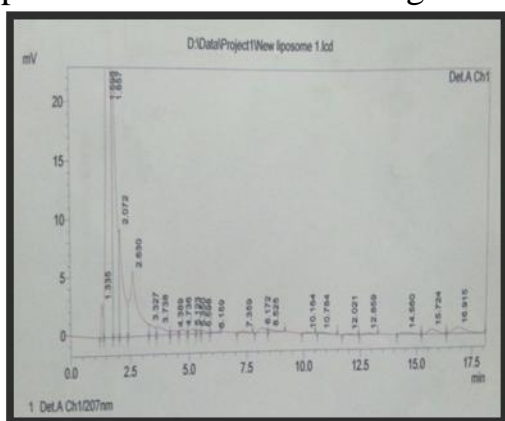
Nanoliposome preparation method	<i>Leishmania</i> Antigen mg/ml nanoliposome	Un-entrapped Antigen (mg/ml)	Absorbance of un-entrapped Antigen	Incorporated Antigen mg/ml	Entrapment Efficiency (% EE)
Exclusion by Sephadex G25	0.4	0.2	0.14	0.2	50
Exclusion by Sephadex G75	0.4	0.29	0.16	0.11	27.5

The amount of drug remaining associated with vesicles was always higher for greater lipid/drug ratios. Mordstrom (13) , found that the EE of liposomes markedly increased from approximately 22% to 65% by increasing lipid /drug ratio from 15 to 30.On the other hand , high lipid

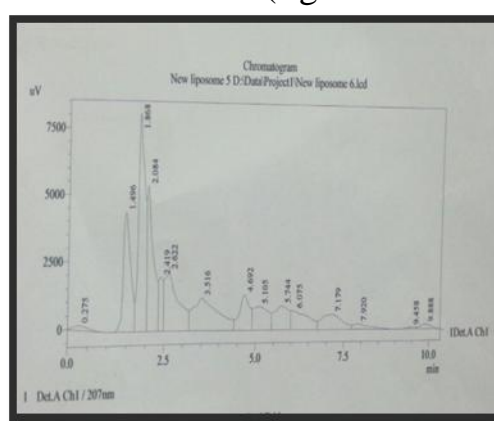
doses may raise concerns of toxicity, reduce the economic feasibility of pharmaceutical scale production and worsen the physical characteristics of the dosage form. Bajelan *et al* (7) found that the strong aggregation , bigger size and solvent used to dissolve the drug have an effect on encapsulation efficiency of nanoparticles , while the solvent have no effect on the release rate . Haeri *et al* (14) were constructed and characterized the liposomal formulation of fluoxetine, as a fourth generation chemosensitizer, and entrapment percent determined . The studied parameters had significant effect on physicochemical characteristics of the nanocarriers. High fluoxetine encapsulation efficiency ( $83\% \pm 3\%$ ) was obtained. 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 .

### Stability of nanoliposomes entrapped SLAs

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). 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 depend on measuring the area of unknown protein in crud soluble antigens at retention time of 5.2 (figures 4 and 5) .



Figure(4):Detection of nanoliposome stability(prepared bySephadex G25) analyzed by HPLC after 10 days of incubation at 4 °C.



Figure(5): Detection of nanoliposome stability (prepared by Sephadex G75) analyzed by HPLC after 10 days of incubation at 4 °C.

Nanoliposomes stability at 4 °C (Figure 6 ) showed decreasing in EE to 27 and 16 % , while stability at 37 °C (Figure7) revealed decreasing in EE to 8 and 5 % within 12 day of storage of nanoliposomes complexes that

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prepared by sephadex G25 and sephadex G75 methods , 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 was highly unstable under physiological conditions(14).

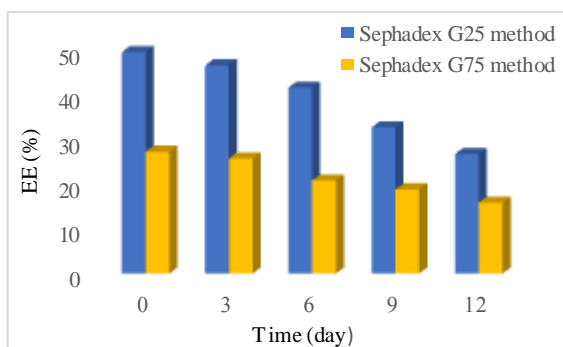


Figure (6) : Stability of SLAs entrapped nanoliposomes at 4°C analyzed by HPLC

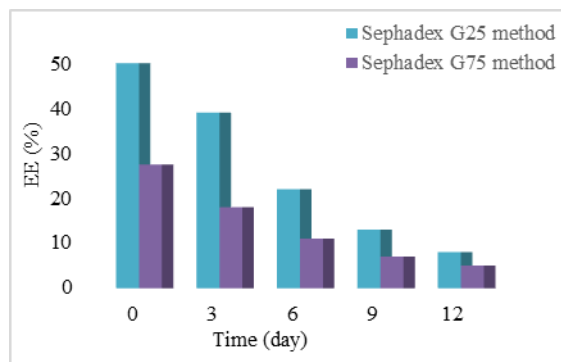


Figure (7):Stability of SLAs entrapped nanoliposomes at 37 °C analyzed by HPLC.

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 .**

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 (%)
Exclusion by Sephadex G25	50	27	46	50	8	84
Exclusion by Sephadex G75	27.5	16	41.8	28	5	82.1



Yang *et al* (8) investigated the stability of fatty acids and vitamin C ( FAs-vit C) entrapped in liposomes at 4 °C for 3 months , and they by presented good stability with relative bigger changes in particle size from  $(86.8 \pm 1.01)$  nm to  $(172.6 \pm 5.51)$  nm . In addition, freeze-dried FAs-vit C complex nanoliposomes experienced little loss of entrapped FAs and vit C during 60 days. These results showed that the FAs-vit C complex liposomes prepared had a favorable stability during storage , suggesting that freeze-drying with certain amount of sucrose had excellent effects for long-term storage of liposomal formulation (Figure 8).

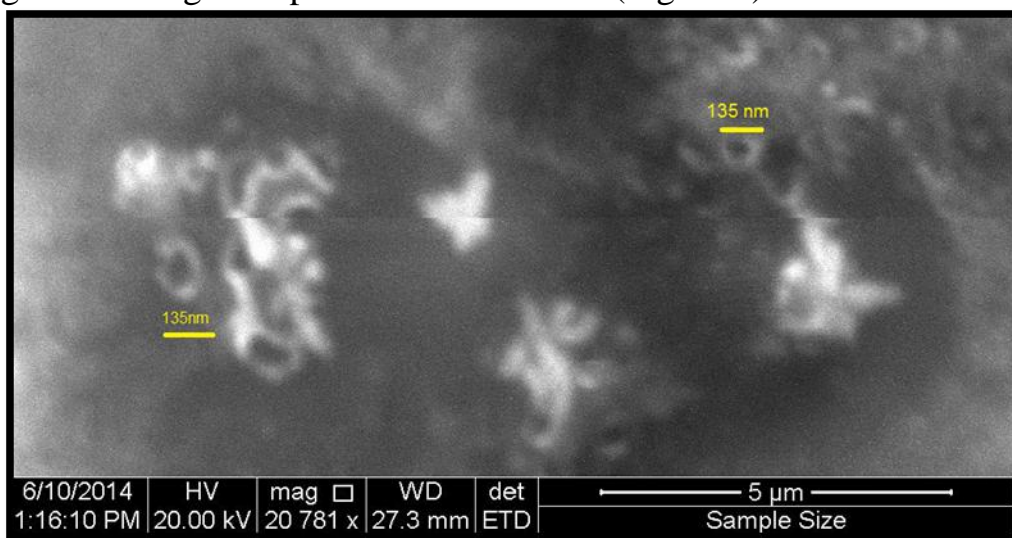


Figure (8): SEM image of degradation of SLAs entrapped nanoliposomes (prepared by Sephadex G25) incubated at 37 °C for 12 days .

The cholesterol appeared to play a role in the in vivo stability of liposomes , we found that the in vitro stability of nanoliposomes entrapped SLAs revealed variable stability at 4 and 37 °C despite the constant concentration of Cholesterol used in both preparing methods . From the results obtained by Haeri *et al* (14), it can be readily noticed that EE, drug release rate and stability of the nanocarrier closely correlated to the liposomal lipid composition.

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# أقتناص مستضدات الشمانيا الاحشائية الذائبة بواسطة الجسيمات الدهنية النانوية المحضرة بطريقة حجم الاستبعاد اللوني

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## الخلاصة

يعتبر استخدام الجزيئات النانوية التي تحمل المستضد و القابلة للتحلل الحيوي كجزئية مساعدة من المواضيع المهمة في تحضير اللقاح ومن الخطوات المهمة في السيطرة على تحرر المستضد لتحسين الاستجابة المناعية. في هذه الدراسة , تم استخدام الجسيمات الدهنية النانوية كمساعدات لقنص المستضدات الذائبة المستخلصة من طفيلي الشمانيا الاحشائية كخطوة اولى باتجاه تحضير لقاح ضد الشمانيا . تم تنشيط الطفيلي من خلال تميمت طور الطفيلي (  $10^4$  ml / promastigote ) في الوسط الزراعي ( RPMI ) في درجة تحضين 23 م<sup>0</sup> ولمدة اربعة ايام . اعتمد اللقاح بعمر 4 ايام وبنسبة  $10^4$  خلية في المليلتر الواحد في تلقيح وسط (SNB9) لانتاج الكتلة الحيوية والتي تستخدم لاستخلاص المستضد . تم استخلاص المستضدات الذائبة من خلايا الطفيلي بعد اربعة تمريرات في نفس الوسط الزراعي ليتم بعدها قنصها في الجسيمات الدهنية النانوية والمحضرة انيا .

حضرت الجسيمات الدهنية النانوية من (4 mM Phosphatidylcholine) ( 2.2 mM Cholesterol ) و ( 0.55 mM Phosphatidylethanolamine ) وبنسبة 1:2:7 ضمن جميع طرق التحضير المختلفة وتم دراسة توصيفها الفيزيائي والكيميائي باستخدام ( SEM ) و ( AFM ) و (FTIR) و ( Zeta Potential ) لتقدير حجم وشكل وشحنة الجسيمات وتحديد المجاميع الكيمائية المتكونة .

تم احتساب كفاءة قنص (EE) الجسيمات الدهنية النانوية للمستضدات الذائبة وكانت النسبة المئوية لكفاءة القنص هي 50 و 27.5 للجسيمات المحضرة بطريقة السيفادكس G75 و G25 على التوالي . من جانب اخر , تم دراسة الثباتية للجسيمات الدهنية النانوية القانصة للمستضدات بدرجاتي حرارة تخزين 4 و 37 م<sup>0</sup>.