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Multi Lamellar Vesicles (Mlvs) Liposomes Preparation by Thin Film Hydration Technique

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ABSTRACT

In the present research the development of liposomes by studying various processes as well as formulation related factors such as effect of cholesterol, effect of alkyl chain length of lipids which may affect the size, shape and encapsulation efficiency of liposomes was reported. Cholesterol and phospholipids were studied by Fourier transform infrared (FTIR) spectroscopy. From scanning electron micrographs, the liposomes appeared in various figures, the sizes of prepared liposomal particles varied in the range of 0.147-0.412 um (147- 412) nm and most of them were multilamellar structures. The vesicle size was found to be varied from 0.147 to 0.412 um. The liposomes were photographed using scanning electron microscope. Vesicles were found to be spherical in shape. Analysis of vesicle size was carried out of each formulation code and the size was found to be reproducible. FT-IR is very useful tool for detecting chemical changes, chemical structures and the thermodynamic of phase transitions and conformational changes in biological systems. Frequency shifts in different regions (symmetric, anti-symmetric stretching or bending, scissoring) or changes in the widths of the corresponding peaks can be used to extract information about various physicochemical processes taking place preparation. This research review the development of liposomes, and speculates optimistically about some future applications.

Keywords: Multi Lamellar Vesicles, Liposomes, Thin Film Hydration

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تحضير كريات اللاييوسومية متعددة الصفائح بواسطة طريقة هدرجة الفلم الرقيق

الخلاصة

نقدم في هذا البحث تقرير عن الجسيمات الشحمية (اللاييوسومات) من خلال دراسة عملية مختلفة، فضلا عن عوامل ذات صلة بالتشكيل مثل تأثير الكولسترول، و تأثير طول سلسلة الألكيل في الدهون التي يمكن أن تؤثر على حجم وشكل و كفاءة التغليف اللاييوسومات. درست نسبة الكوليسترول والدهون الفوسفاتية بواسطة طيف الأشعة تحت الحمراء(FTIR) من المجهر الإلكتروني الماسح، يبدو أن اللاييوسومات في اشكال مختلفة، أحجام متنوعة للاييوسومات المحضرة بمديات 0.147-0.412 مايكرومتر (147-412) نانومتر ومعظمهم ذات متعددة الصفائح. وجد اختلاف في حجم الحويصلة من0.147 الى 0.412 مايكرومتر. تم تصوير الجسيمات الشحمية باستخدام المجهر الإلكتروني الماسح. وجدت حويصلات كروية الشكل. وأجري تحليل لحجم الحويصلة كل تركيب وعثر على حجم تكون متكررة. FT-IR هي أداة مفيدة جدا للكشف عن التغيرات الكيميائية، التراكيب الكيميائية والحرارية والانتقال من مرحلة التغييرات التي تتعلق بتكوين في النظم البيولوجية. انحرافات الترددات في مناطق مختلفة (متماثل ، وامتدادات غير متماثلة أو الانحناء، مقصات) أو تغييرات في عرض القمم التي يمكن استخدامها للحصول على المعلومات حول مختلف العمليات الفيزيائية والكيميائية مع حصول عمليات التحضير. يستعرض هذا البحث تطوير الجسيمات الشحمية،ويخمن بتفاؤل حول بعض التطبيقات المستقبلية.

INTRODUCTION

Liposomes are colloidal, vesicular structures based on phosphor-lipid bilayers. In these structures, an aqueous core is surrounded by lipids arranged in a bilayer configuration. They can be as small as 20 nm and as large as several microns in diameter (Lasic, 1995; Crommelin and Storm, 2003; Crommelin *et al*., 2003; Barenholz, 2003; Felnerova *et al*., 2004; Torchilin, 2005). These vesicles are nontoxic, biodegradable and practically nonimmunogenic (Lasic, 1995, 1996; Felnerova *et al.*, 2004). Because of liposome biocompatibility, they are suitable for every route of administration (Fielding, 1991). They are also used in cosmetics and topical formulations because of their collodial size, easily controllable surface and membrane properties and large carrying capacity (Chen *et al*., 2001). Improved penetration into tissues, especially in the case of dermally applied liposomal dosage forms was reported in several studies. Examples include anesthetics, corticosteroids, and insulin (Lasic, 1995). In the recent years, the topical delivery of liposomes has been used for different applications and in different disease models. After topical application of liposomal formulations, such formulations can significantly increase the rate and extent of drug absorption into epidermis (Fielding, 1991; Lasic, 1995, 1996; Crommelin *et al*., 2003; Barenholz, 2003; Crommelin and Storm, 2003; Torchilin, 2005; Felnerova *et al.*, 2004). Current efforts in this area concentrate on optimization procedures and new compositions (Torchilin, 2005). Liposomes are colloidal, vesicular structures composed of one or more lipid bilayers surrounding an equal numbers of aqueous ompartments. The sphere like shell encapsulated a liquid interior which contain substances such as peptides and protein, hormones, enzymes, antibiotic, antifungal and anticancer agents. A free drug injected in blood stream typically

achieves therapeutic level for short duration due to metabolism & excretion. Drug encapsulated by liposomes achieve therapeutic level for long duration as drug must first be release from liposome before metabolism and excretion (Emanuel *et a*l., 1996). Liposomes are classified on the basis on structural parameters: Unilamellar obvious vesicles (size ranges from 20-1000nm), Oligolamellar vesicles – OLV (These are made up of 2-10 bilayers of lipids surrounding a large internal volume). Multilamellar vesicles – MLV. They can compartmentalize the aqueous volume in an infinite numbers of ways. They differ according to way by which they are prepared. The arrangements can be onion like arrangements of concentric spherical bilayers of LUV/MLV enclosing a large number of SUV etc. While based on method of liposome preparation; REV, Single or oligolamellar vesicles made by Reverse- Phase Evaporation Method. MLV-REV, Multilamellar vesicles made by Reverse-Phase Evaporation Method. SPLV, Stable Plurilamellar Vesicles. FATMLV, Frozen and Thawed MLV. VET, Vesicles prepared by extrusion technique. DRV: Dehydration-rehydration method. Paul Ehrlich in 1906 initiated the era of development for targeted delivery when he envisaged a drug delivery mechanism that would target drug directly to diseased cells, what he called as magic bullets (Alving ,1998; Deamer and Uster, 1980; de Maries *et al*.,1994). of The Liposomes was have many Applications ex. Cancer chemotherapy, Gene therapy, carriers for vaccines and drug in oral treatment, Lysosomal storage disease, Ophthalmic delivery of drugs and others (Vyas & Khar,1996). The aim of recent study to multi-lamellar vesicles (MLVs) liposomes Preparation using thin film hydration technique.

MATERIAL AND METHODS

- **Chemicals**

High purity cholesterol and sigma membrane (12000 MW cut off) were purchased from Sigma Aldrich, Hyderabad, India. Potassium dihydrogen phosphate, disodium hydrogen phosphate, sodium chloride, chloroform, methanol and ethanol were purchased from commercial source. Demonized Distilled Water was used throughout the experiment.

- **Methods**

Multi lamellar vesicles (MLVs) was prepared by thin film hydration technique according to Ramana *et al.* (2007), cholesterol was dissolved in 10 mL solvent system of chloroform and methanol mixture (2:1, v/v) in a 250 mL round bottom flask. The organic solvent system was removed by using rotary evaporator under reduced pressure to obtain a thin film on the wall of the flask. During the process, the conditions such as speed (150 rpm) and temperature 45º ± 2 ºC for conventional liposomes and stealth liposomes were maintained constant. The flask was removed and left overnight in a desiccator under reduced pressure to remove the solvent residuals completely. Then the lipid film was hydrated using phosphate buffer saline pH 7.4 at 60±2 ºC. The resultant suspension was vortexed for about 2 min and a milky white suspension is formed finally. The suspension is allowed to stand for 2 hours in order to complete swelling process, Then the suspension was sonicated using water bath sonicator for about 5 min and extruded through polycarbonate membrane of

0.2µm pore size to give MLVs. Liposomes are generally purified by gel filtration chromatography separation, sephadex-50 is most widely used (Allen and Cleland, 1980; Scherphof *et al*., 1984). To evaluate the liposomes; Liposomal formulation and processing for specified purpose are characterized to ensure their predictable *in vitro* and *in vivo* performance. The characterization of various parameters including size, shape, lamellarity, phase behaviour and drug release profile. Vesicle shape can be assessed using Electron Microscopic Techniques. Vesicle size and size distribution by various techniques These include Light Microscopy, Fluorescent Microscopy, Electron Microscopy (specially Scanning Electron Microscopy), Laser light scattering Photon correlation Spectroscopy, Gel permeation and Gel Exclusion. The most precise method of determine size of liposome is Electron Microscopy since it permit one to view each individual liposome and to obtain exact information about profile of liposome population over the whole range of sizes. Unfortunately, it is very time consuming and require equipments that may not always be immediately to hand (Shashi *et al.*, 2012). The cholesterol and liposomes product was excipients with infrared (IR) grade KBr in the ratio of 1:100 and corresponding pellets were prepared by applying 15000 lb of pressure in a hydraulic press. The pellets were scanned in an inert atmosphere over a wave number range of 4000-500 cm-1 in (Shimadzu, Japan) Fourier transform infrared - FTIR instrument. FT-IR spectra were recorded to the following samples to detect any change in the chemical stability of liposomes due to photo-sensitization of liposomes bilayer after exposure of HYP-ligand liposomes to continuous wave diode laser 650 nm. Scanning electron microscopic analysis was carried out on selected formulations for their morphology (Ramana *et al*., 2007).

RESULTS AND DISCUSSION

From scanning electron micrographs, the liposomes appeared in various figures. The sizes of prepared liposomal particles varied in the range of 0.147-0.412 um (147 - 412) nm and most of them were multilamellar structures as shown in Fig.1. In this study we focused only on thin film method to produce the liposomal particles. We could notice by scanning electron microscopic observation that the thin film method gave unstable shapes of liposomes with different sizes and lamellarity. Many other SEM and Transmission electron microscopy studies also indicated that the particle size, vesicle shape and lamellarity of liposomes may be different due to the process of preparation. As a result the vesicles change their lamellarity, size, size distribution, and shape with time. For example, small vesicles tend to form larger ones and large vesicles smaller ones (Ran and Yalkowsky, 2003; Lautenschlager, 2006; Zasadzinski, 2011). We accepted that scanning electron microscope is a suitable instrument for our research. The results of this study would lead to the next step of research improvement on liposomal preparation to obtain more regularity in shape, size distribution and lamellarity. The vesicle size was found to be varied from 0.147 to 0.412 um (147-412) nm. The liposomes were photographed using scanning electron microscope (Figure 1). Vesicles were found to be spherical in shape. Analysis of vesicle size was carried out for 3 formulations of each formulation code and the size was found to be reproducible.

Table (1) Physicochemical characterization of various Liposome in sampl1.

Liposome sample	Vesicle size, nm	%	Vesicle size um± SE
1	243	20.34	0.243± 0.001
2	177	25.42	0.177± 0.001
3	294	22.03	0.294± 0.012
4	147	8.48	0.147± 0.001
5	412	13.56	0.412± 0.024
6	265	20.34	0.265± 0.022

- The resulted are mean of three replicate.

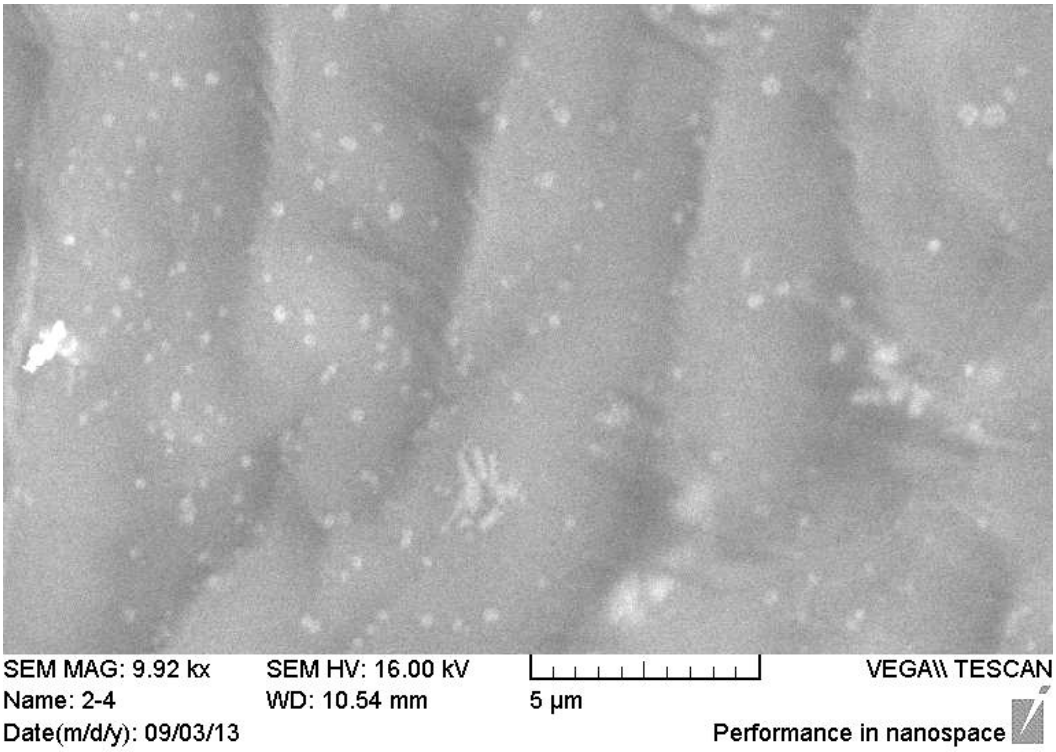

Figure (1) SEM of Sample 1.

Table (2) Physicochemical characterization of various Liposomes in sample2.

Liposome sample	Vesicle size nm	%	Vesicle size um± SE
1	**88**	**20**	**0.088± 0.001**
2	**177**	**20**	**0.177± 0.011**
3	**147**	**60**	**0.147± 0.001**

- The resulted are mean of three replicate.

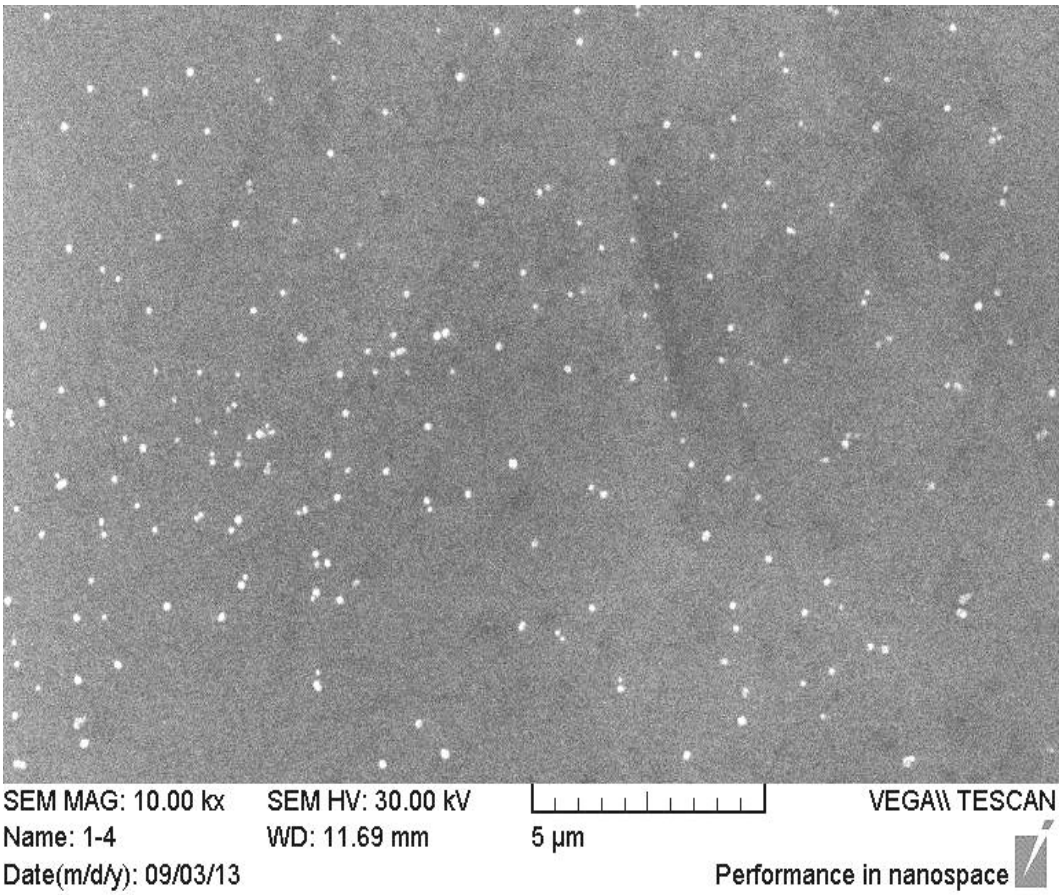

Figure (2) SEM of sample 2 after purification step.

Figure (3) shows the absorption spectra of pure cholesterol compound Spectrophotometric analysis, while Figure (4) shows the absorption spectra of liposomes sample1. FT-IR is very useful tool for detecting chemical changes, chemical structures and the thermodynamic of phase transitions and conformational changes in biological systems before and after preparation. In Figure (3), pure cholesterol has five major peaks at 1022.27 (C-N), 1188.15 (C-H wag –CH2X, 1381.03 (C-h rock…alkanes), 1465.9 (C-H bend) and 3412.78 (N-H stretch) nm. These four peaks shifted to 1120.94 (C-O stretch), 1226.73 (C-O stretch), 1365.6 (C-H rock), 1419.61 (C-C stretch… in-ring), 1465.9 (C-H bend) and 1645.28 (N-H bend) and 3417.88 (O-H stretch, H-bonded) nm in liposome sample after preparation. The high base line is a consequence of light scattering. The infrared spectra of lipids have been studied in detail and most bands have been assigned (Feride *et al*., 1995). Various kinds of information can be derived from these bands. Frequency shifts in different regions (symmetric, anti-symmetric stretching or bending, scissoring) or changes in the widths of the corresponding peaks can be used to extract information about various physico-chemical processes taking place. For example, the frequencies of the CH_2 stretching bands of acyl chains depend on the average trans/gauche isomerization in the system. The shifts to higher wave numbers correspond to an increase in number of gauche conformers (Feride *et al*., 1995). Bandwidth gives dynamic information about the system. As the bandwidth increases, mobility of the phospholipids acyl chains increases. Figures (3, 4) represent FT-IR absorption spectra of control sample (cholesterol), liposome sample1, respectively.

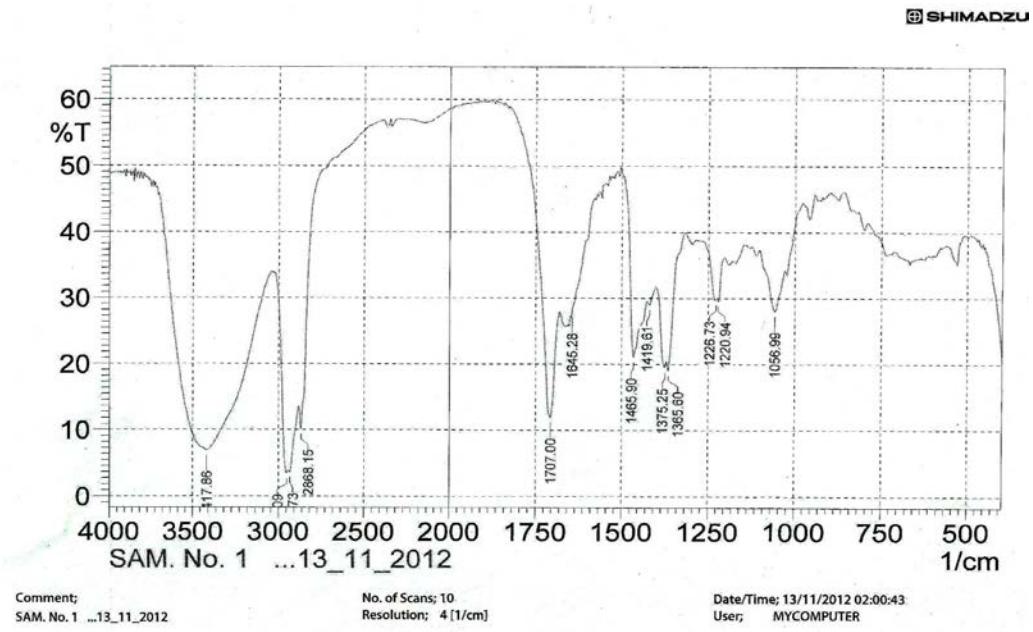

Figure (3) FTIR spectrum of pure cholesterol.

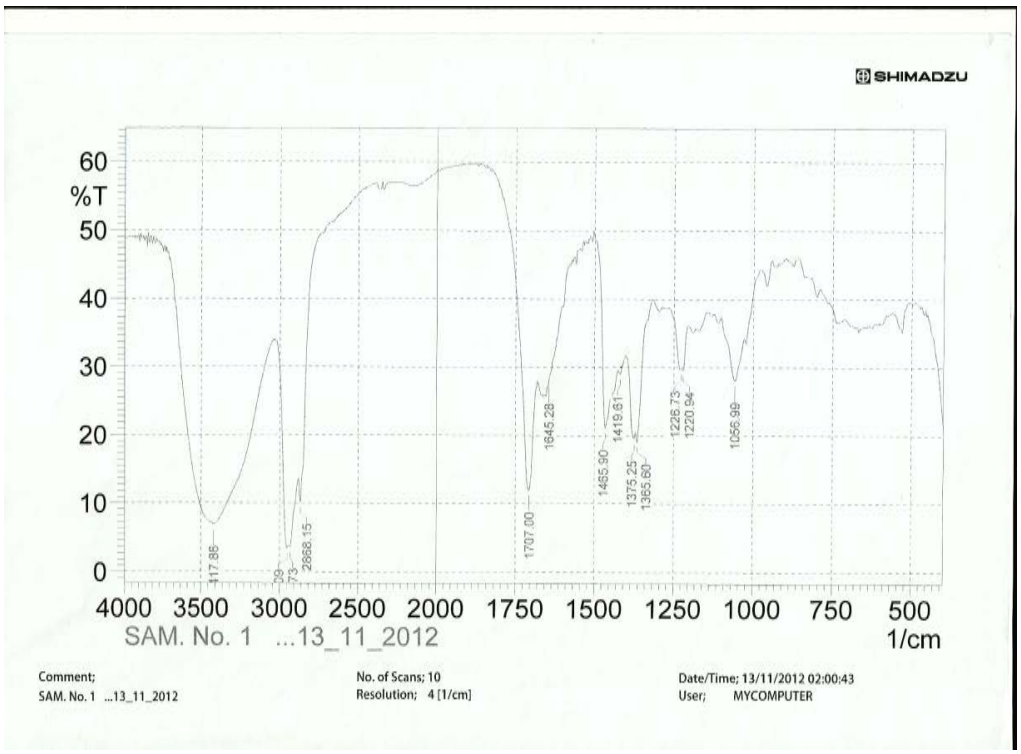

Figure (4) FTIR of liposome sample.

Liposomes are generally purified by gel filtration chromatography separation; Sephadex-50 is most widely used. Purification of liposomes when lipids are routinely carried out via gel permeation chromatography, an extremely lengthy procedure, and in the method we report, this lengthy step was replaced by the use of molecular-weight cut-off filters. Using this novel method, large unilamellar vesicles were produced and the time required, post-rehydration, was dramatically reduced from almost 48 to less than 2 hours, with a highly uniformly sized population of liposomes being produced- the homogeneity of the liposome population achieved using our method was 99%, as compared to 88% attained by using the traditional method of production.

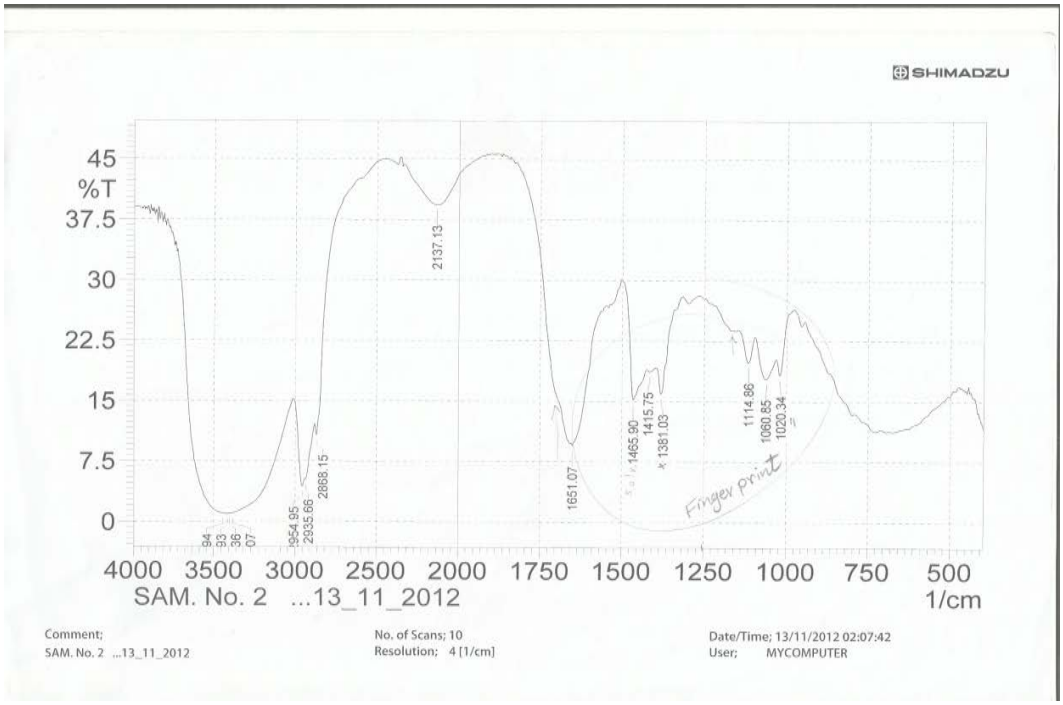

Figure (5) FTIR of liposomes sample2.

Liposome stability can be subdivided into physical, chemical and biological stabilities, which are all inter-related. The shelf-life stability of pharmaceutical and cosmetic products is determined by the physical and chemical stability of the liposomes (uniformity of size distribution and encapsulation efficiency, and minimal degradation of all compounds, respectively). By optimizing the size distribution, pH and ionic strength, as well as the addition of antioxidants and chelating agents, liquid liposome formulations can be stable for years (Alving, 1998).

Our data provide some insight into the physical and chemical mechanism of liposome membrane stabilized. FT-IR analyses were accomplished on C-H, C=O and choline group (N-$(CH_3)_3$) of phospholipids of liposomes For OH-group, control sample has broad peak at 3465.46 cm^{-1} and lower wave number shoulder at 3434.60 cm^{-1} Figure(5). These peaks were shifted to longer wave number for sample (I) after exposure to laser. The CH_2-stretching (symmetric and anti-symmetric) modes at 2841 and 2922 cm^{-1} respectively of control sample(I), but in the Figure(5) appear increased when exposure to laser to in sample(I) at 2874 and 2955 cm^{-1} respectively Figure (5). These shifts toward higher frequencies indicate on increasing proportion of gauche to Tran's conformers, consequently, the mobility of the lipid acyl chains would be increased (Constanca and Dirk, 2006). The C=O band of control sample (I) with narrow band at1735 was shifted up field (longer wave number) to 1745 cm^{-1} for sample (I) after exposure to laser. This shift is attributed to broken down of hydrogen

bonds between C=O of acyl chains of liposomes. Choline group of control sample (I) has asymmetric stretching vibration peak on 1025 cm^{-1} which is higher than of standard value of pure lipid (at 970 cm^{-1}). This peak was shifted down field for sample (I) and liposomes at 863 cm^{-1} due to breaking down this hydrogen bond. All these changes in FT-IR results between control (I) and sample (I) result in increasing permeability of liposomes to the encapsulated drug. Liposomes are biocompatible, completely biodegradable, non-toxic and non immunogenic. Suitable for delivery of hydrophobic, amphipathic and hydrophilic drugs. Protect the encapsulated drug from the external environment. Reduced toxicity and increased stability-As therapeutic activity of chemotherapeutic agents can be improved through liposome encapsulation. This reduces deleterious effects that are observed at concentration. Similar to or lower than those required for maximum therapeutic activity. Reduce exposure of sensitive tissues to toxic drugs (Danilo, 1996; Immordino *et al.*, 2006). In the present study we report development of stealth liposomes by studying various process as well as formulation related factors such as effect of cholesterol, effect of alkyl chain length of lipids which may affect the size, shape and encapsulation efficiency of liposomes. Cholesterol and phospholipids were studied by Fourier transform, infrared (FTIR) spectroscopy. We conclude the vesicle size of liposomes was found to be varied from 147 to 412 nm, and the vesicles were found to be spherical in shape and the FT-IR is very useful tool for detecting chemical changes.

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