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Liposomes as Amphiphilic Carriers: Encapsulation and Stability Aspects

(Liposom sebagai Pengangkut Amfifili: Aspek Pengkapsulan dan Kestabilan)

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ABSTRACT

The aimed of the present study was to evaluate the liposomal formulation regarding its hydrophobicity. The evaluation studies were done based on the amphiphilic nature of the phospholipid liposomes. This paper highlights the importance of such type of lipid based carriers by encapsulation hydrophobic and hydrophilic drug models. Crystal violet and Nile red were used to represent hydrophilic and hydrophobic moieties before moving to pharmaceutical implications. The formulated liposomes were compared for their hydrophobicity using percent encapsulation efficiencies. The purpose of this formulation was to mimic the red blood cells. The average particle size of 120±25.1 and zeta potential of -10.2±1.4 were in good agreement with reported characteristics of the red blood cells. Per cent encapsulation efficiency for crystal violet was more obvious with a value of 68.1 as compared to 36.5% for Nile red. The prepared liposomes were quite stable for a period of one month. Our findings reflect the fate of our system more suitable for hydrophilic drugs under the given set of formulation parameters.

Keywords: Crystal violet; drug hydrophobicity; Nile red; percent encapsulations; phospholipid carriers

ABSTRAK

Matlamat kajian ini adalah untuk menilai penggubalan liposomal mengenai kehidrofobiannya. Kajian penilaian telah dijalankan berdasarkan sifat amfili daripada liposom fosfolipid. Kertas kerja ini membincangkan kepentingan apa-apa jenis penerbangan yang berpangkalan lipid oleh hidrofobi pengkapsulan dan model dadah hidrofili. Kristal ungu dan Nil merah telah digunakan untuk mewakili moieti hidrofili dan hidrofobi sebelum berpindah ke implikasi farmaseutik. Formulasi liposom dibandingkan untuk kehidrofobiannya menggunakan suatu kecekapan pengkapsulan peratus. Tujuan pembentukan ini adalah untuk meniru sel darah merah. Purata saiz zarah 120±25.1 dan *potensi zeta daripada -10.2±1.4 adalah sama dengan ciri yang dilaporkan untuk sel darah merah. Peratus kecekapan pengkapsulan untuk kristal ungu adalah lebih jelas dengan nilai sebanyak 68.1 berbanding 36.5% bagi Nil merah. Liposom yang disediakan agak stabil untuk tempoh satu bulan. Penemuan kami menunjukkan sistem kami lebih sesuai untuk dadah hidrofili dengan parameter formulasi yang diberikan.*

Kata kunci: Dadah kehidrofobiannya; kristal ungu; Nil merah; pembawa fosfolipid; peratus pengkapsulan

INTRODUCTION

Proper designs of drug delivery systems, its monitoring and evaluating strategies regarding specific targets have been very crucial in obtaining accurate stability and controlled drug release studies (Chen et al. 2014). In view of the stability and targeted delivery aspects, controlled average particle size (Karewicz et al. 2013), optimum lipid compositions (Mosca et al. 2011), drug loading strategies (Hou et al. 2013; Rossi et al. 2004), released kinetic studies (Hou et al. 2013; Millan et al. 2004) have played a significant role for the last few eras. This is done in order to enhance the collective inter-related effects of physical, chemical and biological stabilities of nano carriers especially liposomes (Ashraf et al. 2015; Krisenko et al. 2014; Zhu et al. 2013). Special attention has been given to liposomes as preferred drug delivery systems based on their amphiphilic nature (Cao et al. 2010; Kastner et al. 2015). The inability to deliver drugs to the intended biological targets has long been a major obstacle in drug delivery systems. Liposomes are believed to offer minimum toxic side-effects by offering shielding effects for both hydrophilic and hydrophobic drugs of interest (Dyondi et al. 2015; Krasnici et al. 2003; Zhou et al. 2014). The liposomes are the vesicles having the ability to accommodate hydrophilic drugs in internal aqueous cores and the hydrophobic drugs in the lipid bilayer. The lipid bilayer protects the drug degradation on the way to targeted sites during circulation. Toxicity risks using liposomes are reduced which is a common practice for frequently used chemotherapeutics. Cisplatin and vincristine are among the examples which offer peripheral neurotoxicity and anthracyclines cardiotoxicity (Dimitriu et al. 2014; Rafiyath et al. 2012; Surhio et al. 2014). Liposomal delivery of chemotherapeutics like nausea and vomiting resulted in notably mild or even absence of any side effects which were generally associated with routine

chemotherapy treatments (Lee at al. 2010; Raymond et al. 2011). In treatment of kaposi's sarcoma (Raimundo et al. 2013) and ovarian carcinoma patients (Kaye et al. 2012), DaunoXome and Doxil have been approved as liposomal-based drugs in the present context (Dawidczyk et al. 2014; Szebeni et al. 2012). Ligands may also be accommodated for target based nanotechnology using liposomes. Being amphiphilic, both hydrophilic and hydrophobic drugs can be retained within the liposomes until they are reached the targeted side well in time.

This study reports that the overall stability of our liposome formulation will not only be related to phospholipid moieties, special attention has been given to the encapsulation efficiencies of the liposomes. The formulation of liposomes was done in order to mimic the red blood cells (Hincha 2008; Merkel et al. 2011). The reported average particle size and zeta potential of the red blood cells is 80 to 150 nm and -9 to -15 mV, respectively (Chapanian et al. 2012; Huang et al. 2011). The study was conducted to check the hydrophobicity of the system using model dyes prior to any drug encapsulation. The encapsulation ability of the liposomes was compared for both hydrophilic and hydrophobic drugs. In order to determine the effect of lipid composition on the liposome stability and drug loading capacity, crystal violet and Nile red was used as model hydrophilic and hydrophobic drugs respectively (Cottenye et al. 2013; Manca et al. 2012). The chemical structures of the dyes are given in Figure 1. We have used lecithin, Dipalmitolphosphatidylcholine (DPPC) (Elliott et al., 2005, Schaffran et al., 2010), Distearolyphosphatidylcholine (DSPC) (Lajunen et al. 2015; Mfuh et al. 2011), phosphatidylserine (PS) (Dimitriu et al. 2014; Ran et al. 2015) and dicetyl phosphate (DCP) (Batool et al. 2015; El-Ridy et al. 2015; Sasaki et al. 2013) for liposomes formulation. The system developed was compared for its hydrophobicity using these model dyes with reference to its average particle size, zeta potential and per cent encapsulation efficacies.

Materials and Methods

L-α-phosphatidylcholine from Soybean (CALBIOCHEM), Chloroform (Merck Sdn Bhd (Petaling Jaya, Selangor, Malaysia), Dipalmitolphosphatidylcholine (DPPC), Distearolyphosphatidylcholine (DSPC), phosphatidylserine (PS), dicetyl phosphate (DCP) and phosphate buffer saline (PBS) tablets (sigma-aldrich) were used. All the solutions and samples were prepared using deionized water (Barnstead Diamond™ RO unit, Barnstead International, USA). Further purification of chloroform was done by distillation followed by nitrogen gas purging in order to avoid oxidation of the lecithin.

Preparation of Liposome

Multilamellar liposomes were prepared by thin film hydration method described by Bangham in 1965. A definite quantity of lecithin was mixed with equal ratios of phospholipids and DCP using chloroform as solvent medium. The mixture was subjected to the rotary evaporator (50°C) followed by dispersion of resulted thin film with PBS dispersion medium (warmed at 50°C in water bath). The dispersion medium was 0.01M PBS at pH7.4.

Crystal violet and Nile red were encapsulated in the above formulated liposomes in a different way. Being hydrophobic, Nile red was dissolved in chloroform together with the lipid mixture. Crystal violet was dissolved in the phosphate buffer saline dispersion medium as per its hydrophilic nature. Thin film was then hydrated with the same medium later on.

Liposome Sizing

Multilamellar liposomes were obtained as a result of thin film dispersion in PBS. These were then subjected to probe sonicator for proper sizing purpose. The sonicator was set at a total of 5 min process time. It was also further set at 10 s pulse on and 15 s pulse off time for getting nano size liposomes.

Average Particle Size, Zeta Potential and Polydispersity Index Measurement

The liposomes were analyzed for their size, zeta potential and polydispersity index using a Malvern NanoSeries ZetaSizer (Malvern, UK) at a constant temperature of 25°C.

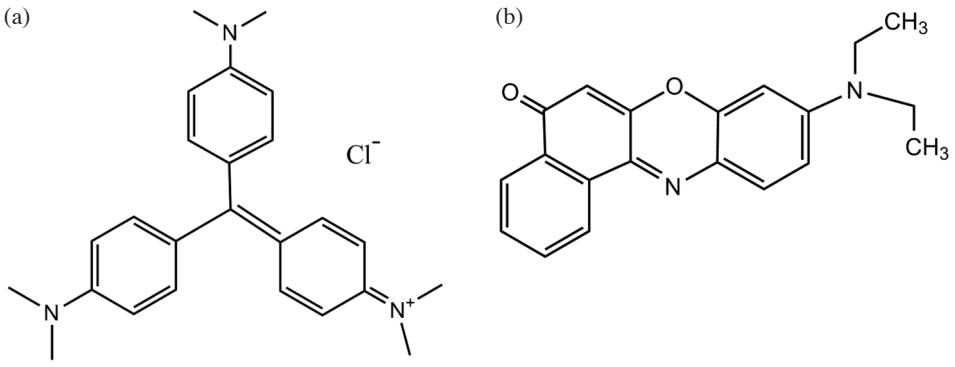

FIGURE 1. Chemical structures of crystal violet (a) and Nile red (b)

TRANSMISSION ELECTRON MICROSCOPY

The prepared formulation was subjected to an energy Filtered TEM model LIBRA 120 (quipped with an Olympus SIS-iTEM, version 5) to study morphology of nanosized liposomes. The procedure followed involved the placement of one drop of formulation onto a carbon-coated copper grid. A filter paper was used to remove the excessive amount of formulation after 1 min. A negative stain, 2% phosphotungstic acid, was applied on the grid for only 1 min. The excessive stain was also removed with the help of a filter paper. The grid was air dried initially and then was put in desiccator for five days before being examined under transmission electron microscope.

CALIBRATION CURVES FOR CRYSTAL VIOLET AND NILE RED

The calibration curves for nile red and crystal violet were prepared by making different standard solutions from pure compounds. The solutions were subjected to the UV/VIS spectrophotometer for absorbance measurements. The absorbance readings for crystal violet and Nile red were taken at λ max 589 and 537 nm, respectively.

ENCAPSULATION EFFICIENCY (% EE)

In order to determine the encapsulation efficiency of loaded liposomes, the loaded liposomes were subjected to ultrafiltration method. The liposomes were encapsulated with crystal violet and Nile red for determining the hydrophobicity of the liposome formulation under study. 1 mg of each dye was loaded to compare the encapsulation efficiency using centrifugation process for 30 min at about 9000 rpm (Vivaspin 6, Sartorius Stedim Biotech, Germany). The amount of encapsulated dyes was determined by taking supernatant of the centrifuged liposomes using Spectrophotometer (Cary 50 UV-VIS Spectrometer, Agilent Technologies, USA). The concentration of respective dyes in each sample was determined from standard calibration curves. The effect of centrifugation intensity was also noted. Per cent encapsulation efficiency was calculated using the following:

$$\%EE = \frac{WT - WF}{WT} \times 100 \quad (1)$$

where WT is the total amount of dyes added; and WF is the amount of unloaded dyes.

STABILITY STUDIES

Stability of the liposomes encapsulating crystal violet and Nile red was determined by keeping them in dark at 37°C over a time period of one month. The stability studies were done with respect to average particle size, zeta potential and the polydispersity index using Malvern Zeta Sizer.

RESULTS AND DISCUSSION

LIPOSOME DESIGN AND CHARACTERIZATION

The neutral head group present on the phosphotidylcholine molecules (PC) is known for stability of liposomes, however, negatively charged lipids also contribute towards this aspect according to previously reported studies. As a consequence, a combinatorial liposome approach has been followed since eras involving phospholipids with negatively charged head groups. The examples may include head groups such as phosphatidylglycerols (PG) to neutral phosphatidylcholine (PC) mixtures. Therefore, the present study also involves the negatively charged phospholipids as well. Liposomes were prepared from two different model dyes with varying hydrophobicity/hydrophilicity. Water crystal violet has been found to have water solubility of 16 g/L at 25°C (Buckley et al. 2015; Dai et al. 2015). In contrast, water solubility values for Nile red have been reported as very slightly soluble in water (< 1 ug/mL) indicating its hydrophobic nature (Kurniasih et al. 2015; Ramireddy et al. 2015). The comparative systematic study of these two representative dyes allowed to investigate encapsulation efficiencies with reference to drug hydrophobicity. Liposome samples were prepared and characterized using Malvern Zeta Sizer. The samples were found to have the same size range of 120±26 nm, allowing a relatively valid stability comparison between the systems under study (Huang et al. 2011). The zeta potential and the polydispersity index were obtained as -9.6±2.1 and 0.5, respectively (Table 1). The focus was to mimic red blood cells, therefore, the attempts were made to attain a zeta potential of red blood cells i.e. -9 to -15 mV.

MORPHOLOGY OF LIPOSOMES USING TEM

TEM images of the pure unencapsulated liposomes showed almost a spherical shape as shown in Figure 2. The small nanosized particles were in agreement with the Malvern zeta particle sizer, i.e. around 100 to 120 nm. TEM micrographs are shown in the figure with a single liposome in the inset with a scale bar of 100 nm.

ENCAPSULATION EFFICIENCY

The loaded liposomes were subjected to UV/Vis Spectrophotmeter analysis regarding encapsulation

TABLE 1. Composition of the liposome for encapsulation of model dyes

Liposome composition (mg)	Average particle size (nm)	Polydispersity index	Zeta potential (mV)
Lecithin: DPPC: DSPC: PS: DCP (70:1:1:1:30)	120±25.1	0.416±0.01	-10.2±1.4

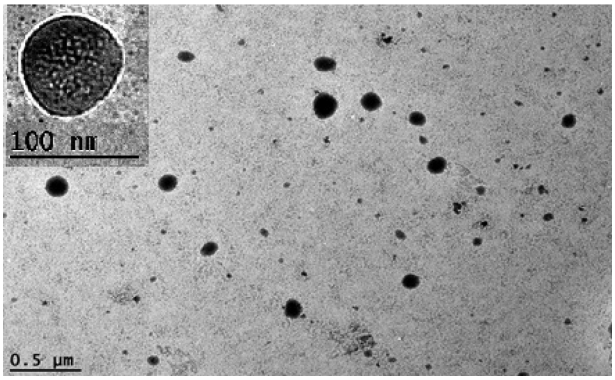

FIGURE 2. Transmission electron microscopic image for the liposomes obtained at a scale of 0.5 µm (500 nm). The inset shows a single liposome at a scale of 100 nm

efficiency measurements. The loaded liposomes were analyzed for encapsulation efficiency using standard calibration curves (Figure 3). The wavelengths used were 590 and 537 nm for crystal violet and Nile red, respectively. The supernatant from each sample was taken out at different speed of centrifuge machine like 2500, 5000, 7500, 10000 and 12500 rpm. This was done in order to check the optimum speed for such liposomal suspensions. It was found that the absorbance values against each sample were increasing until almost a constant value for the last two speeds. The reason for this increase in absorbance value was attributed to the fact that low speeds of centrifuge were not sufficient to separate suspended liposomes from its fluid mediums (Khaskheli et al. 2015; Ming et al. 2015). It showed that the 10000 rpm may be used to centrifuge the liposomes with such lipid compositions. The encapsulation efficiency for both samples was measured with reference to the relevant standard calibration curves. The results showed that encapsulation efficiency for crystal violet was high in comparison with Nile red i.e 36 and 68.9% (Figure 4). This difference in the encapsulation led to a conclusion that the system developed for liposome formulation is well suited for the hydrophilic drugs more than the other. The Nile red is more likely to be entrapped in the lipophilic region of the liposomes owing to its very low water solubility (Wu et al. 2015). On a contrary, the high water solubility of crystal violet let it be stored predominantly in the central aqueous compartments of the liposomes (Bennett et al. 2015; Papancea et al. 2015). This difference in potential storage locations render them sensitive where stability discussions are concerned as reported in previous literature.

The greater the phospholipid tail movements the greater are the expulsion of bilayer stored contents compared to the cargo stored in its cores. Therefore, literature also reports greater release of hydrophobic moieties reflective of the interplay between the physical

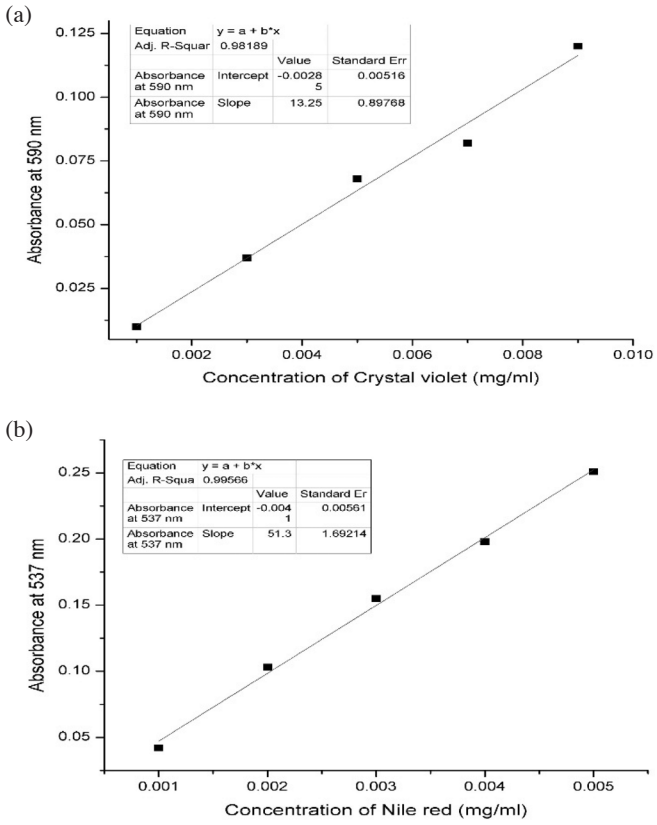

FIGURE 3. Standard calibration curves for crystal violet (a) and Nile red (b) using UV/VIS spectrophotometer

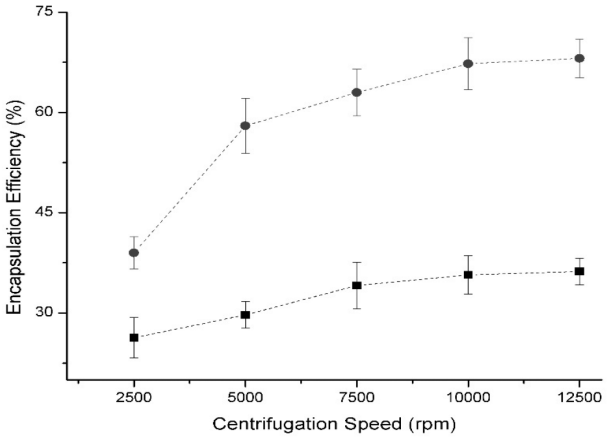

FIGURE 4. Effect of centrifugation speed on the encapsulation efficiency of crystal violet (●) and Nile red (■)

properties of the cargo and phospholipid liposome compositions (Manca et al. 2012; Parente-Pereira et al. 2014).

CONCLUSION

The present study has focused on the comparative approach of the liposome loading characteristics with model drug cargos as a function of average particle size, zeta potential, polydispersity index, stability and encapsulation efficiencies. Liposomes with major phospholipid contents were found to be comparatively stable over a time period of one month at 37°C. Hydrophilic drugs have been found to be well suited for the liposome formulation under study as compared to the hydrophobic drugs. Systematic correlations of delivery efficiencies for unique physical properties of liposomes can also account for a 'tunable' approach for drug transport and other applications.

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