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Physicochemical evaluation of DEAE-Dx coated liposomes for long alkyl chain lipid

Premanarayani Menon, Yin Yin Teo* and Misni Misran

The purpose of this work was to study the potential of diethylaminoethyl dextran (DEAE-Dx) coated liposomes as drug carriers. Thin film hydration method was employed to prepare 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-distearyl-sn-glycero-3-phosphocholine (DSPC). The critical vesicular concentration (CVC) of DPPC and DSPC were found to be 0.08 % (w/v) and 0.06 % (w/v), respectively. As stability is a general problem with liposomes, DEAE-Dx as a polymer was used to promote steric stabilization by coating the surface of the DPPC and DSPC liposomes. Liposomes stabilized by DEAE-Dx were superior to the corresponding non-coated liposomes. The surface behaviour of DPPC and DSPC was investigated through surface tension analysis before and after the addition of DEAE-Dx. All the liposomes were evaluated based on their particle size, zeta potential and morphology. A thirty-five day stability study shows that the particle size and zeta potential of DEAE-Dx coated liposomes were stable at room temperature. The DEAE-Dx coated liposomes were loaded with an antihistamine drug, diphenhydramine hydrochloride (DPH). The encapsulation efficiency profile shows that DEAE-Dx coated DPPC (DPPC-DEAE-Dx) liposomes have higher entrapment of DPH compared to DEAE-Dx coated DSPC liposome (DSPC-DEAE-Dx). An in vitro release experiment demonstrated DEAE-Dx coated liposomes had the best controlled release system.

Introduction

Liposomes are artificially constructed spherical vesicles composed of phospholipids or amphiphatic lipids enclosing water or aqueous buffers. Liposomes are made up of material similar to a cell membrane; therefore, they represent an advanced vehicle for transport of substances such as nutrients and pharmaceutical drugs into the cell, out of the cell and between different parts of a cell [1]. Throughout the years, the use of liposomes has substantially advanced medical applications in drug delivery owing to their excellent properties such as biocompatibility, biodegradability, non-toxicity and flexibility. In the dermatological field, liposomes were initially preferred as a drug carrier due to its moisturizing and restoring properties. Later, other applications of liposomes were explored such as the ability to encapsulate various types of drug as well as administration of these drugs to the epidermal cells and deeper cell layers [2]. Because of their amphiphilic structure, liposomes can entrap hydrophilic drug in their aqueous compartment and lipophilic drugs within the lipid membrane [3]. Also, they have the ability to protect and sustain the release of the encapsulated drug.

The formulation of an appropriate drug carrier liposomal system depends on the composition of the liposomes. The nature of the lipids composing the liposomes affects the membrane fluidity, charge density, steric hindrance and permeability of the vesicles [4]. In general, naturally-derived phospholipids such as egg phosphatidylcholine (Egg PC) and soy phosphatidylcholine (Soy PC) with varying fatty acyl chain compositions are used to prepare the lipidic vesicles. However, the behaviour of these naturally derived lipids, which exist as a mixture are difficult to control and are thus less preferred for medical application, especially for intravenous administration of drugs while synthetic or semi-synthetic lipids which comprise of only one lipid species are more likely preferred for medical applications [5]. Another issue often related to liposomes is their stability. Unmodified liposomes, regardless of whether composed of synthetic lipids or naturally derived lipids, result in aggregation of the vesicles which subsequently causes the leakage of encapsulated material [6].

To address the issue of liposome stability, we have incorporated a polycationic polymer diethylaminoethyl dextran (DEAE-Dx) in the formulation process in order to promote the stability of liposomes. We have studied the effect of DEAE-Dx coating on liposomes composed of lecithin, which is a mixture of naturally derived lipids, in our previous work [7]. In the present study, long alkyl chain lipids such as DPPC (16 alkyl chain length) and DSPC (18 alkyl chain length) were used to prepare the liposome with the purpose of investigating the variation
in physicochemical characteristics arising from different alkyl chain lengths. Surface tension studies were performed to determine the CVCs of DPPC and DSPC solutions as these values have not been clearly reported elsewhere. Also, the effect of DEAE-Dx on the surface tension and CVC was reported herein in order to elucidate the interaction of DEAE-Dx polymer with lipids in the bulk solutions. Polymer coating of liposomes is considered as a robust technique to enhance the stability of liposomes [8]. DEAE-Dx was shown to fulfill many of the requirements for a good coating material, comparable to other commonly used polymers such as polyvinyl alcohol [9], chitosan [10], pectin [11] and carboxymethyl dextran [12]. Another polymer that is widely used for its stabilizing effect is polyethylene glycol (PEG). Unfortunately, PEG has to be modified or functionalized with active ligands in order to facilitate steric stabilization on liposomes, which involves a higher production cost [13]. On the other hand, preparation of DEAE-Dx coated liposomes involves a simpler technique that simply requires mixing of a liposome suspension with DEAE-Dx solution therefore resulting in a lower production cost compared to pegylation of liposomes. Most importantly, DEAE-Dx is biodegradable and biocompatible [14].

The encapsulation efficiencies and in vitro drug release of a hydrophilic drug, diphenhydramine hydrochloride (DPH), from DPPC-DEAE-Dx and DSPC-DEAE-Dx liposomes were also studied. DPH is an antihistamine used to treat severe allergic symptoms such as itchiness, common cold, insect bites and bee stings. DPH is effective in the treatment of allergic skin disorders because it distributes into the skin efficiently and sustains higher concentrations than in serum. Unfortunately, high dosage administration of DPH has side effects such as sedation and drying of mouth, which limits its usage [15]. Also it has been reported that administration of high dosages of DPH may increase sensitivity to sun-light, eventually leading to skin rash and sunburn [16]. The main objective of this study is to develop a formulation with stable DPPC-DEAE-Dx and DSPC-DEAE-Dx liposomes followed by encapsulation and in vitro release of DPH. The present study suggests that liposome-encapsulated DPH will attain a prolonged delivery, thereby reducing the potential dose-related side effects.

Experimental

Materials

1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and diethylaminoethyl dextran (DEAE-Dx) (average molecular weight of 500,000, degree of substitution corresponds to one DEAE-substituent per three glucose units) were purchased from Sigma-Aldrich (St. Louis, USA). Diphenhydramine hydrochloride (DPH) purchased from Cayman Chemical (Michigan, USA). Chloroform of emprove grade was obtained from Merck. All samples were prepared using deionized water with a resistivity of 18.2 $\Omega$ cm$^{-1}$.

Surface tension measurements of DPPC and DSPC solutions

DPPC or DSPC (0.14 % w/v) was dissolved in chloroform and the solutions were dried under reduced pressure by using a rotary evaporator (Model Buchi Rotavapor R114, Switzerland) which results in the formation of thin layer lipid film on the wall of the flask. The thin film was hydrated and gently shaken at a temperature above the gel to liquid crystalline phase transition temperature ($T_c$) using warm deionized water. The surface tensions of DPPC and DSPC solutions were determined by the Du Noüy ring method using a Force Tensiometer (Model Sigma 702, Finland) at 30.0 °C. The critical vesicular concentration (CVC) was determined from the inflection point of a graph of surface tension vs. natural logarithm of the concentration of lipid in solution. Similar procedures were applied to measure the surface tensions of the DPPC-DEAE-Dx and DSPC-DEAE-Dx mixtures while keeping the DEAE-Dx concentration constant and varying the lipid concentration. The effect of increasing DEAE-Dx concentration from 0.02 % (w/v) to 0.08 % (w/v) on the surface behaviour of the lipid was also investigated.

Preparation of DPPC and DSPC liposomes

DPPC liposomes were prepared at a concentration of 0.4 % (w/v). DPPC was dissolved in chloroform and the solutions was dried under reduced pressure by using rotary evaporator which results in the formation of thin layer of lipid film on the wall of the flask. For non-encapsulated DPPC liposomes, warm deionized water of 5 ml was added to the flask. The dry films for DPH encapsulated DPPC liposomes were hydrated with 5 ml of DPH solution with concentrations ranging from 0.03 % (w/v) to 0.19 % (w/v). The solutions were subjected to sonication using a bath type sonicator to obtain uniformly sized particles. A similar procedure was applied for the preparation of 0.4 % (w/v) DSPC.
Coating of DPPC and DSPC liposomes with DEAE-Dx

Liposomes coated with various concentrations of DEAE-Dx were prepared by slowly adding 0.4 % (w/v) DPPC or DSPC liposomes into equal volume of DEAE-Dx solution (0.01, 0.02, 0.03, 0.04 and 0.05 % w/v) during magnetic stirring. The mixture was stirred for approximately 1 hour at room temperature. The resulting mixture solution contained 0.2 % (w/v) of DPPC or DSPC and 0.005, 0.01, 0.015, 0.02 or 0.025 % (w/v) DEAE-Dx. DEAE-Dx coated liposomes containing encapsulated DPH were prepared in the similar manner with addition of DPH ranging from 0.015 % (w/v) to 0.095 % (w/v).

Transmission electron microscopy (TEM)

The morphology of uncoated and DEAE-Dx coated liposomes were obtained by using transmission electron microscope (TEM) (Model JEOL JEM-2100F, Japan) after storage period of 5 days. A drop of liposomal suspension was placed onto a carbon-coated copper grid, and the excess solution was drawn off with filter paper. The sample was then negatively stained using 2 % (w/v) phosphotungstic acid solution and air-dried at room temperature before TEM measurement.

Particle size and zeta potential measurement

The average hydrodynamic particle size and zeta potential of the liposome and DEAE-Dx coated liposome solutions were measured using Malvern NanoSeries Zetasizer (Malvern Instrument, UK) at a constant temperature of 25 °C. The stability of the uncoated and DEAE-Dx coated liposomes were monitored over a period of 35 days at room temperature.

Encapsulation efficiency of DEAE-Dx coated liposome

The percentages of DPH incorporated in DPPC and DSPC liposomes were determined by a centrifuge method (Model Eppendorf 5804R Centrifuge) [13]. The DPH-loaded liposome was centrifuged at 10 000 rpm for 60 minutes in order to separate entrapped DPH in the liposome from the free DPH. The absorbance of free DPH in the clear supernatant was then determined using a ultraviolet-visible (UV-Vis) spectrometer (Cary 50 UV-Vis spectrometer, Agilent technologies, USA) at wavelength of 258 nm. The concentration of DPH was determined from a calibration curve constructed from standard solutions (0.01 mg/ml – 0.12 mg/ml). The DPH encapsulation efficiencies of liposomes were calculated as in equation 1

\[
EE (\%) = \frac{T - S}{T} \times 100 \quad [\text{Equation 1}]
\]

Where EE is the encapsulation efficiency, T is the theoretical concentration of DPH added and S is the concentration of DPH detected in supernatant.

In vitro drug release

In vitro drug release of uncoated and DEAE-Dx coated liposomes were evaluated by using the Automated Franz Diffusion Cell System (Microette Autosampling System, Hanson Research Co., USA) with 0.636 cm² of effective diffusion area. The receptor compartments were filled with 4 mL of 10 mM phosphate buffered saline (PBS) solution (pH 7.4) and continuously stirred at a constant speed of 400 rpm while the temperature was equilibrated at (37 ± 1) °C. Regenerated cellulose membranes with a 5000 Da molecular weight cut-off were sandwiched between the donor and receptor compartments. The membranes were pretreated before being mounted to Franz Diffusion Cells by soaking in the receiving medium overnight. Each sample of about 1 mL was introduced into the donor compartments. Receptor phase samples were withdrawn at predetermined intervals throughout the 24 h experimental period and were refilled with fresh receiving medium to maintain a constant volume. The drug content at each interval was obtained spectrophotometrically.

Results and Discussion

Surface tension

CVC is an important quantitative parameter determined via the inflection point from a plot of surface tension (γ) versus ln[lipid]. The value of CVC reflects the minimum concentration of lipid required to form liposomes, which also implies the tendency of lipids to self-assemble. When lipids dissolve in water, they adsorb and orientate at the water surface so that the hydrophobic regions are removed from the aqueous environment. The replacement water molecules by lipids at the surface causes a reduction in the surface tension due to weaker intermolecular forces between lipid and water molecules compared to those between two water molecules [17]. By comparing the γ of DSPC and DPPC, the γ of DSPC was found to be lower than DPPC (Fig. 1). This result indicates that DSPC has higher surface activity compared to DPPC owing to the fact that it has a longer alkyl chain that promotes the hydrophobicity of the molecule. On the other hand,
the CVCs of DPPC and DSPC were determined to be 0.08 % (w/v) and 0.06 % (w/v) respectively. DSPC, which has a longer alkyl chain, poses a stronger hydrophobic force compared to DPPC; thus lower the concentration of DSPC required to form liposomes [18]. This study implies that DPPC and DSPC at concentrations above 0.08 % (w/v) and 0.06% (w/v), respectively, tend to self-assemble into vesicles. Additionally, it is important to study the CVC of liposomes to avoid preparation liposome solutions that have either too high or too low of concentration for physicochemical studies [19].

The changes in the γ behaviour of DPPC and DSPC after the addition of DEAE-Dx were also studied (Table 1). It was observed than upon addition of DEAE-Dx, the γ of DPPC and DSPC increased. The changes in the γ profile were mainly attributed to the presence of DEAE-Dx. These results suggest that DPPC and DSPC bind to DEAE-Dx respectively, thereby promoting the removal of lipid from the surface into the bulk phase. Therefore, in the presence of DEAE-Dx, fewer lipids are required for the formation of vesicles. This phenomenon clearly explains the reason for the slight decrease of CVC upon addition of DEAE-Dx. The Gibbs equation was employed in order to explore how the area, occupied by DPPC molecule and DSPC molecule at air/water interface changes upon addition of DEAE-Dx. The equilibrium between surfactant molecules at the surface of the solution and those in the bulk of solution is expressed by Gibbs equation as described by Equation 2 and 3.

\[
A = \frac{1}{N_A} \Gamma_{\text{max}} \quad \text{[Equation 2]}
\]

\[
\Gamma_{\text{max}} = \frac{-1}{nRT} \left( \frac{d\gamma}{d\ln C} \right) \quad \text{[Equation 3]}
\]

Where \(N_A\) is the Avagadro’s number, \(\Gamma_{\text{max}}\) is the surface excess concentration, \(n\) is the number of molecules species involved, and \(\frac{d\gamma}{d\ln C}\) is the gradient of the plot of surface tension against \(\ln C\).

The area occupied by DPPC was smaller compared to area occupied by DPPC-DEAE-Dx. A similar result was observed for DSPC and DSPC-DEAE-Dx. These results imply that without addition of DEAE-Dx, the lipid monomers were more closely packed at the air/water interface. It can be deduced that in the presence of DEAE-Dx, the arrangement of lipid monomers is disrupted, thus leading to an increase in the area occupied at the air/water interface. Furthermore, an increase in concentration of DEAE-Dx exhibited some effect on the γ profiles of DPPC and DSPC. When DEAE-Dx concentration was increased from 0.02 % (w/v) to 0.04 % (w/v), the surface tension increased slightly and the CVC value was reduced. However, upon further increase to 0.08 % (w/v) DEAE-Dx had no significant effect on the γ of DPPC and DSPC. From these results, it can be understood that a DEAE-Dx concentration of 0.04 % (w/v) was sufficient to bind to lipid monomers and remove the lipid monomer from surface to bulk phase.

Table 1: Surface behaviour of DPPC and DSPC after addition of DEAE-Dx

<table>
<thead>
<tr>
<th>Sample</th>
<th>Without DEAE-Dx</th>
<th>0.02 % DEAE-Dx</th>
<th>0.04 % DEAE-Dx</th>
<th>0.08 % DEAE-Dx</th>
<th>Without DEAE-Dx</th>
<th>0.02 % DEAE-Dx</th>
<th>0.04 % DEAE-Dx</th>
<th>0.08 % DEAE-Dx</th>
</tr>
</thead>
<tbody>
<tr>
<td>CVC % (w/v)</td>
<td>0.08</td>
<td>0.05</td>
<td>0.04</td>
<td>0.04</td>
<td>0.06</td>
<td>0.04</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>Surface tension</td>
<td>59.7</td>
<td>63.7</td>
<td>64.3</td>
<td>64.8</td>
<td>58.2</td>
<td>63.4</td>
<td>64.9</td>
<td>65.0</td>
</tr>
</tbody>
</table>

Particle size of DEAE-Dx coated liposomes

The effects of DEAE-Dx addition on the hydrodynamic diameter of DPPC and DSPC liposomes was investigated with DEAE-Dx concentrations ranging from 0.005 % (w/v) to 0.025 % (w/v) (Fig. 2). Concentrations of both lipids were fixed at 0.2 % (w/v), which is above their respective CVC values. The particle size of DSPC liposomes was found to be (99 ± 2) nm with a polydispersity index of 0.4 whereas DSPC liposomes had a particle size of (72 ± 1) nm with polydispersity index of 0.2 before the addition DEAE-Dx. Addition of DEAE-Dx up to 0.020 % (w/v) resulted in an increase of the hydrodynamic diameter of the DPPC and DSPC liposome to (140 ± 1) nm and (122 ± 1) nm, respectively, with a polydispersity index of 0.4 in both cases. The increase in the size of liposomes indicated the interaction between DEAE-Dx and liposomes. However, upon addition of 0.025 % (w/v) of DEAE-Dx, there was no significant change in the liposomal size compared to liposomes coated with 0.02 % (w/v) DEAE-Dx. This indicated that surface of the liposome were saturated with DEAE-Dx. Overall, DSPC liposomes had a smaller particle size compared to DPPC liposomes. It is expected that DSPC with additional of two carbon atoms in the alkyl chain have a stronger hydrophobic force compared to DPPC; thus lower the concentration of DSPC required to form liposomes [18]. This study implies that DPPC and DSPC at concentrations above 0.08 % (w/v) and 0.06% (w/v), respectively, tend to self-assemble into vesicles. Additionally, it is important to study the CVC of liposomes to avoid preparation liposome solutions that have either too high or too low of concentration for physicochemical studies [19].
Zeta Potential of DEAE-Dx coated liposomes

Zeta potential is a parameter that has been used to investigate the stability of a colloidal system. Particles in suspension with larger magnitudes of zeta potential have a greater tendency of repulsion between particles, and thus more stable is the suspension [13]. The variation in the magnitude of zeta potential of DPPC and DSPC liposomes after coating with DEAE-Dx is shown in figure 3. DPPC and DSPC, which are pure lipids, had relatively low magnitudes of zeta potential; however, addition 0.005 % (w/v) DEAE-Dx to DPPC and DSPC liposomes resulted in a significant increase of zeta potential value from +1.5 mV and +0.6 mV to +20.7 mV and +23.7 mV, respectively. The tremendous increase in magnitude of zeta potential after addition of DEAE-Dx suggests the successful coating of positively charged DEAE-Dx on the surface of the lipids. It was also observed that as the concentration of DEAE-Dx was increased to 0.02 % (w/v), the magnitude of the zeta potential of DPPC and DSPC liposomes also increased to +30.6 mV and +33.7 mV, respectively. Further increase of DEAE-Dx to 0.025 % (w/v) did not modify the magnitude of the zeta potential of either DPPC or DSPC liposomes significantly. Overall, DSPC liposomes had slightly higher magnitudes of zeta potential compared to DPPC liposomes indicating that DSPC liposomes, which are of smaller particle size, have greater mobility and present greater steric repulsion than DPPC liposomes.

Evaluation of stability for uncoated and DEAE-Dx coated liposome

The entire sample was kept for a period of 5 days before TEM analysis. Surface morphological studies using TEM on the shape of DPPC and DSPC liposomes indicated that the particles were almost spherical. The uncoated DPPC and DSPC liposomes were larger in size compared to the DEAE-Dx coated liposomes under the same preparation condition after a period of 5 days (Fig. 4). The morphological behaviour of the liposomes was in agreement with the result obtained from the stability of particle size measurement (Fig. 5) in which the uncoated liposomes exhibited increase in size with a long time scale typically days compared to the DEAE-Dx coated liposomes.

The change in particle size of uncoated and DEAE-Dx coated liposomes were analysed in order to investigate the colloidal stability of these liposomes which were kept at 30 °C over a period of 35 days (Fig. 5). After 7 days of storage, both uncoated DPPC and DSPC liposomes had shown drastic increase in particle size. After 35 days of evaluation, the particle size of uncoated DPPC liposome continued to increase from (99 ± 2) nm to (721 ± 4) nm whereas particle size of uncoated DSPC liposome increased from (72 ± 1) nm to (451 ± 1) nm. The obtained results suggest the aggregation of the particles into clusters at a significant rate and clearly show that uncoated DPPC and DSPC liposomes are not stable. However, the extent of particle size increase in DSPC liposomes was slightly lower compared to DPPC liposomes over a period of 35 days. The relative stability of DSPC is likely due to the fact that it contains longer alkyl chain that causes stronger attraction between the chains and thus results in more closely packed chain compared to DPPC [21]. Closely packed chains, in turn, have lower tendency for aggregation and subsequent bilayer disruption due to their strong cohesion force. Additionally, curvature effects on lipid packing in liposomes also affect the stability of liposomes. Longer alkyl chain lipids form liposomes with smaller particle size due to their higher curvature. This phenomenon supports the rational where DSPC, with smaller particle size, possesses higher repulsive force in the solution and hence has lower tendency to form aggregates compared to DPPC liposomes. As displayed in figure 5, the particle size of DEAE-Dx coated DPPC and DSPC liposomes displayed very little change throughout the storage period, suggesting that DEAE-Dx coating enhances the colloidal stability of DPPC and DSPC liposomes.

Figure 6 demonstrated the changes of zeta potential of uncoated and DEAE-Dx coated liposomes, which were monitored over 35 days. A relatively lower magnitude of zeta potential is observed for uncoated DPPC and DSPC liposomes compared to the coated liposomes and their zeta potential values fluctuated over the storage period. On the other hand, the zeta potentials of coated DPPC and DSPC liposomes were observed to increase after storage of 7 days and were stabilized after 35 days of evaluation. The same phenomenon was also observed in our previous work in the evaluation of zeta potential of DEAE-Dx coated lecithin liposomes. As mentioned in our previous work, this phenomenon is due to the slow adsorption of polymer on the liposomes from solution [22]. Hence, the coating and uncoiling of the DEAE-Dx polymer on the surface of the liposomes during the first 7 days may explain the increase in zeta potential. After 7 days, the adsorption of the polymer onto the surface of the liposomes achieved equilibrium and therefore a constant zeta potential was observed between 7 and 35 days of evaluation. Additionally, the coated DPPC and DSPC liposomes had larger magnitudes of zeta potential compared to the uncoated ones; thus, these particles had greater repulsion between particles which contributed to a lower tendency toward aggregation. Therefore, the increase in magnitude of the zeta potential value by coating of DEAE-Dx on the liposomal surface can be attributed to the improvement in the stability of the liposomes. This phenomenon supports the particle size results over 35 days, in which the coated liposomes exhibited little variation in particle size. Overall, 0.02 % (w/v) DEAE-Dx coated DPPC liposomes and 0.01 % (w/v) DEAE-Dx coated DSPC liposomes were observed to be the most stable dispersion as they show very little changes in particle size and zeta potential.
potential over a period of 35 days. Thus, these formulations were chosen to study the encapsulation efficiency and release of encapsulated drugs.

### Encapsulation of Diphenhydramine Hydrochloride

The optimum concentration of DPH that can be incorporated into liposomes composed of DPPC-DEAE-Dx and DSPC-DEAE-Dx was investigated. It can be observed that at very low concentrations of DPH, the encapsulation efficiency is also low, while increasing the concentration of DPH leads to higher encapsulation efficiency (Fig. 7). The reason that DPH concentration affects the encapsulation efficiency is due to high solubility of DPH in the bulk medium. Therefore, at low concentrations of DPH, the probability of DPH being entrapped in liposomes is lower and is expected to be higher as the concentration of DPH increases. However, once the liposomes are saturated with DPH, further increase in DPH causes a reduction of encapsulation efficiency. DPPC-DEAE-Dx liposomes show optimum encapsulation at 0.065 % (w/v) DPH with an efficiency of 37.2 % whereas DSPC-DEAE-Dx liposomes show optimum encapsulation at 0.055 % (w/v) DPH with an efficiency of 36.1 %. The slightly higher encapsulation efficiency of DPPC-DEAE-Dx liposomes shows that DPPC liposomes have a greater trapping volume, as the particles are larger in size compared to DSPC liposomes [23]. This result agrees with the particle size measurement in which the hydrodynamic diameter of DPPC liposome is bigger compared to DSPC liposomes. It can be deduced that size of liposomes is correlated with its encapsulation efficiency.

#### In vitro drug release

DPPC-DEAE-Dx and DSPC-DEAE-Dx liposomes with 0.065 % (w/v) and 0.055 % (w/v) of DPH were selected as the optimized formulation based on the results obtained from the encapsulation efficiency analysis. The cumulative DPH release from DPPC-DEAE-Dx and DSPC-DEAE-Dx liposomes was investigated. Also, the release of free DPH and the release of DPH from uncoated DPPC liposomes and DSPC liposomes were investigated in order to study the effect of DEAE-Dx coating on the release of DPH. Cumulative release was plotted against time for all samples as shown in figure 8. For the first 2 hours, there was not much difference in the rate of release for the liposomal systems compared to free DPH solutions. This could be due to the presence of free DPH molecules in the aqueous phase that diffuse rapidly through the membrane. However, the release rate of the liposomal systems began to slow down as compared to the free DPH solution after 2 hours. This phenomenon suggests the retention of DPH in the liposomes, causing a slower rate of diffusion through the membrane.

Overall, this study showed that cumulative DPH release from liposomal systems was lower compared to the free DPH solution containing same DPH concentration over a 24 hours release profile.

This study also revealed that coating of liposomes with DEAE-Dx could prolong the drug release. For the first 8 hours, coated and uncoated DPPC liposomes released almost the same amount of DPH; however, for the next 16 hours, coated DPPC liposomes exhibited slower release compared to the uncoated liposomes. The same phenomenon was observed for DSPC liposomes, in which coated liposomes had slower release for the subsequent 16 hours compared to uncoated ones. The presence of DEAE-Dx could protect the liposomes against drug leakage by retaining the drug in the liposomes for a longer time. Sustained release of DPH from liposomal system could help to reduce the adverse side effects of DPH.

We also compared the in vitro release of DPH from DPPC-DEAE-Dx and DSPC-DEAE-Dx liposomes respectively (Fig. 9). DSPC-DEAE-Dx liposomes shows slightly slower cumulative release per area compared to DPPC-DEAE-Dx liposomes. The plausible reason is that DSPC, which bears a longer alkyl chain, has a higher phase transition temperature compared to DPPC due to stronger van der Waals forces between the lipid chains [24]. Thus, higher energy is required to disrupt the packing of DSPC bilayers, hence slowing down the release of the DPH.

The in vitro release of four samples was curve fitted to zero-order, first-order, Higuchi and Korsmeyer-Peppas models by DDSolver software to understand their release kinetics (Table 1). The model with the highest correlation coefficient was considered to be the best model. Correlation coefficient values were high in all cases but the Korsmeyer-Peppas model was found to be the best fitting model. The values of the diffusion exponents, n, were found more than 0.43 and less than 0.89, which indicates a Fickian release. Accordingly, model. Correlation coefficient values were high in all cases but the Korsmeyer-Peppas model was found to be the best fitting model. The software to understand their release kinetics (Table 1). The model with the highest correlation coefficient was considered to be the best.

### Table 2: Different kinetic model evaluation of DPH release for four different samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Zero Order</th>
<th>First Order</th>
<th>Higuchi</th>
<th>Korsmeyer-Peppas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Slope</td>
<td>R²</td>
<td>Slope</td>
<td>R²</td>
</tr>
<tr>
<td>DPPC</td>
<td>3.74</td>
<td>0.93</td>
<td>0.06</td>
<td>1.00</td>
</tr>
<tr>
<td>DPPC-DEAE-Dx</td>
<td>3.39</td>
<td>0.87</td>
<td>0.06</td>
<td>0.98</td>
</tr>
</tbody>
</table>

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Please do not adjust margins
DSPC & 4.22 & 0.94 & 0.08 & 0.99 & 17.26 & 0.95 & 10.06 & 0.99 \\
DSPC-DEAE-DX & 3.76 & 0.91 & 0.07 & 0.99 & 15.47 & 0.95 & 9.98 & 0.99 \\

### Conclusions

DPPC and DSPC liposomes coated with DEAE-Dx were found to be more stable than uncoated liposomes. Furthermore, the preparation process for DEAE-Dx coating on liposomal surfaces reported herein is simple and straightforward, and hence has the potential to be used in the bulk production of liposomes. Additionally, the effect of longer alkyl chain DSPC (18 alkyl chain length) with higher hydrophobicity leads to smaller value of CVC, hydrodynamic size, lower encapsulation efficiency and slower in vitro release of DPH compared to DPPC (16 alkyl chain length). This study also supported the hypothesis that liposome encapsulated DPH will attain a prolonged drug delivery. Interestingly, slow release was better exhibited by DEAE-Dx coated liposomes compared to the uncoated ones.

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### References

Fig. 1. Surface tension profile (a) and schematic illustration on the surface condition (b) of DPPC and DSPC liposomes solution.

Fig. 2. Variation in the size of DEAE-Dx coated liposomes with varying concentration of DEAE-Dx.
Fig. 3. Variation in zeta potentials of DEAE-Dx coated liposomes with varying DEAE-Dx concentration.

Fig. 4. TEM micrographs of liposomes (a) uncoated DPPC liposomes, (b) 0.02 % (w/v) DEAE-Dx coated DPPC liposomes, (c) uncoated DSPC liposomes, (d) 0.01 % (w/v) DEAE-Dx coated DSPC liposomes over a period of 5 days.
Fig. 5. The influence of DEAE-Dx concentration on the particle size of (a) DPPC liposomes and (b) DSPC liposomes over a period of 35 days.

Fig. 6. The influence of DEAE-Dx concentration on the zeta potential of (a) DPPC liposomes and (b) DSPC liposomes over a period of 35 days.
Fig. 7. Encapsulation Efficiency of DEAE-Dx coated DPPC and DSPC liposomes with varying concentration of DPH.

Fig. 8. *In vitro* release of (a) free DPH, uncoated and coated DPPC liposomes and (b) free DPH, uncoated and coated DSPC liposomes over a period of 24 hours.
Fig. 9. *In vitro* release comparison of DEAE-Dx coated DPPC liposomes DEAE-Dx coated DSPC liposomes over a period of 24 hours.