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Original Article

Effect of Bilayer Flexibility and Medium Viscosity on Separation of Liposomes upon Stagnation

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Abstract

Liposomes are widely used as drug delivery systems in different forms including osmotic pumps, infusion and IV injection. In spite of these, there is no data available about their behavior under convective flow (e.g. infusion or osmotic pumps) and upon stagnation in such drug delivery systems. As a part of a series of investigations in this area, the present study investigates the effects of viscosity and flexibility on liposomes separation upon stagnation.Here, liposomes with different bilayer flexibility and medium viscosity were encountered gravity (separating force) in a designed sedimentation model and changes in their properties were monitored over time. Rigid liposomes in the low viscosity formulation showed significant phase separation (three times reduction in size) and decreased lipid content over time. Increasing the bilayer flexibility of large liposomes, prevented them from phase separation. Neither size reduction nor decreased lipid content was observed. Increasing viscosity of the liposomal formulation of 3.4 cP to 45.2 cP also prevented sedimentation of liposomes and phase separation in the system. These results indicate that bilayer flexibility and viscosity affect the separation of large liposomes in pre-administrational steps and even stagnation during administration in systems such as infusion pumps.

Keywords: Bilayer flexibility, Liposomes, Particle size, Sedimentation, Separation, Stagnation, Viscosity,

1. Introduction

Liposomes are of great interest as an advanced carrier-based drug delivery system. Liposomal formulations are used in multiple areas, from clinical application of the liposomal drugs to the development of various multifunctional liposomal systems for therapy and diagnostics [1]. The special properties of liposomes have generated numerous applications of liposomes as drug delivery

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system [2] which is feasible with various drug molecules and routes of administration and as models for biological membranes [3]. The application of liposomes are growing rapidly as complex and sophisticated liposomal formulations such as dendrosomes [4, 5] and magnetoliposomes [6, 7] and prolonged-release gel-based liposomal systems [8, 9] are being introduced in the field. The stability of liposomes is a major consideration in all steps of their production and administration: from process steps to storage to delivery. When a pharmaceutical dosage form is altered the stability of the drug may be changed. A stable dosage form maintains its physical integrity and does not adversely influence the chemical integrity of the active ingredient during its life on the shelf [10]. Different instabilities such as chemical and physical are probable to occur. One of these instabilities is changes in particle size distribution of liposomal formulations. Exact knowledge of the sizes of these nanoparticles is essential because, size can substantially affect physicochemical and biopharmaceutical behavior of the formulation. For example, variations in particle size can affect drug release kinetics, transport across biological barriers, and pharmacokinetics in the human body [11]. This study focuses on the influence of physical factors in size separation of liposomes –a physical instability- as a respond to gravitational force during stagnation.

Particles size distribution of liposomes can change in stagnating conditions such as: in storage and mixing tanks, intravenous infusion pumps or drip, osmotic or physically actuated pumps and etc. Size separation of particles encountering gravity can be affected by their physical characteristics and the media they are dispersed in. Such problems can worsen by introduction of novel complex liposomal such as dendrosomes [4, 5] and magnetoliposomes [6, 7] that are expected to be heavier due to their heavy loads. Viscosity and flexibility also seem to affect phase separation of liposomes in short term stagnation. Some studies reported medium viscosity of and shape of particles (which is affected flexibility) to be effective on particles sedimentation. [13]. Despite of these reports, there is no data available about the effect of bilayer fluidity and medium viscosity of on separation of liposomes in pre-administrational stagnation. As a part of series of studies aimed to investigate the behavior of liposomes in convective flow and stagnation, this article investigates the influence of the bilayer flexibility of liposomes and medium viscosity of on their separation during stagnation. Such stagnations are possible in liposomal drug delivery systems both before administration (e.g. during storage and pre-administration steps) and during administration (e.g. in a running infusion

pump). There is not such a data available in the literature.

2. Material and Methods

2.1. Materials

Egg phosphatidylcholine (EPC, >80%) and 1, 2-distearoyl-sn-glycero-3-phosphocholine (DSPC, >99%) were purchased from Lipoid GmbH (USA), cholesterol (>99%) and propylene glycol (PG) was purchased from Sigma (USA). methanol (99%), chloroform (99-99.4%), ammonium thiocyanate (98.5%) and ferric chloride hexahydrate (>99%) were purchased from Merck (Germany). All other analytics were of pharmaceutical grade.

2.2. Preparation Liposomal Formulations

These liposomal formulations were prepared by lipid film hydration method. The lipids were dissolved in chloroform: methanol mixture (2:1) and the organic solvent was evaporated at 60°C under vacuum to form a lipid film using a rotary evaporator (Heidolph, Germany). The film was then hydrated by deionized water at the same temperature. Total lipid content of liposomes was 20 mM.

To investigate the effects of viscosity and bilayer flexibility, different liposomal formulations were prepared here. Rigid low viscosity liposomes were formulated using DSPC: cholesterol (70:30 molar ratios). To prepare high viscosity liposomes, water was replaced with propylene glycol. EPC: cholesterol (50:50) was used to obtain more flexible liposomes

Fluidity of liposomes depends on their lipid composition and phase transition temperature of phospholipids. EPC with Tm of about 0°C and DSPC with Tm of about 55°C [14] were used to obtain liposomal formulations with different bilayer flexibilities. In this direction, EPC: cholesterol (50: 50) was used as flexible liposomes and DSPC: cholesterol (70: 30) was used as rigid liposomes.

Extrusion (Northern Lipids, Canada) of liposomes was conducted to adjust their size. Size of all three liposomal formulations reduced using the same extrusion process to obtain same size distribution in different formulations. Liposomes were extruded 10 times through polycarbonate filters with pores size of 1μm.

2.3. Characterization of Liposomes

2.3.1. Measuring the Size Distribution and Zeta Potential

Size distributions of the particles were measured using Mastersizer 2000 (Malvern, UK). Charges of the nanoparticles were also analyzed by measuring the zeta potential of the liposomal formulations, using Nanozetasizer (Malvern, UK). $D_{0.5}$ (diameter which size of the 50% of the particles is less than) was also measured.

2.3.2. Measurement of Phospholipid Content of Liposomes

As an indicator of liposomes concentration, the concentration of phospholipids was measured using the Stewart Method [15]. This method uses the reaction between phosphate group of phospholipids and ammonium ferrothiocyanate to measure the concentration of phospholipid in the samples. In brief, a

proper amount of liposome was dissolved in chloroform and was shaken vigorously in the presence of ammonium ferrothiocyanate. The concentration of phospholipid the chloroform was then measured using UV-Visible spectrophotometer (CECIL, Italy) at 488 nm after separation of chloroform from aqueous phase.

2.4. Phase Separation Studies

Possible separation of liposomes upon stagnation was investigated in burettes in a vertical position using the gravity as the driving force, as described earlier (the article is under press at International Journal of Pharmaceutical Research). To perform this investigation, liposomal formulations were first put in separate burettes and after a settling time of 15 hours, samples were collected in defined time interval for 10 hours. Changes in the formulations during the experiment were then analyzed using size distribution profile, $D_{0.5}$ and lipid contents of samples.

2.5. Statistical Analysis

Statistical analysis of the results was performed by one-way ANOVA test using the SPSS Statistics 17.0 software (SPSS Inc., USA).

3. Result and Discussion

3.1. Characterization of Liposomes

The particle size distribution curve of rigid low viscosity liposomal formulation showed two peaks at 200 and 1100 nm with particle range between 30-2000 nm. Zeta potential of this formulation was measured to be -1.38 mV (Figure 1) and its $D_{0.5}$ was measured to be about 450 nm with a span of 3.1 (n=3).

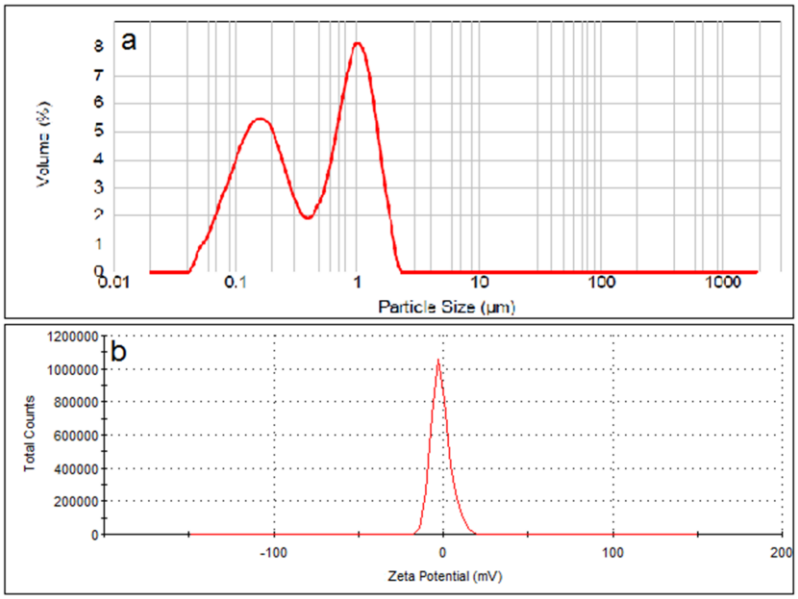

Figure 1. Particle size distribution (a) and zeta potential (b) of low viscosity rigid liposomes.

The flexible liposomes showed a $D_{0.5}$ of about 270 nm with a span of 4.9 (n= 3). Range of size distribution in this formulation was from 40 nm to 2000 nm. The size distribution

curve contained two peaks at 80 and 900 nm. Analyzing of the zeta potential of these particles confirmed that they carry a negative charge of about -20 mV (Figure 2).

Particle size of high viscosity liposomal formulation showed $D_{0.5}$ of about 700 nm with a span of 1.8 (n=3). Size distribution curve of these particles contains two peaks at 150 and 900 nm that covers 40-2000 nm. Zeta potential of the particles measured to be about 0 mV (Figure 3).

3.2. Effect of Bilayer Flexibility

Lipid composition and phase transition temperature (Tm) of lipids determine the fluidity of liposomes. To control bilayer

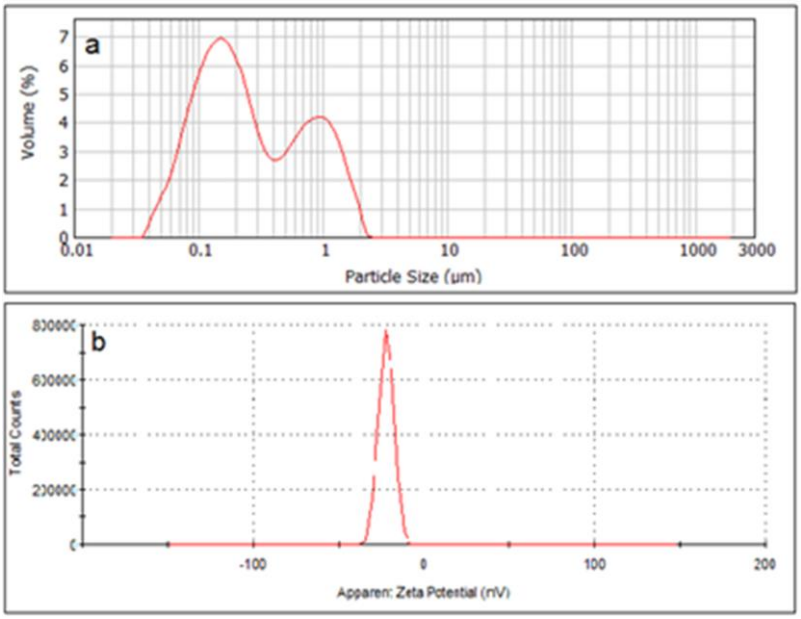

Figure 2. Particle size distribution (a) and zeta potential (b) of flexible liposomes.

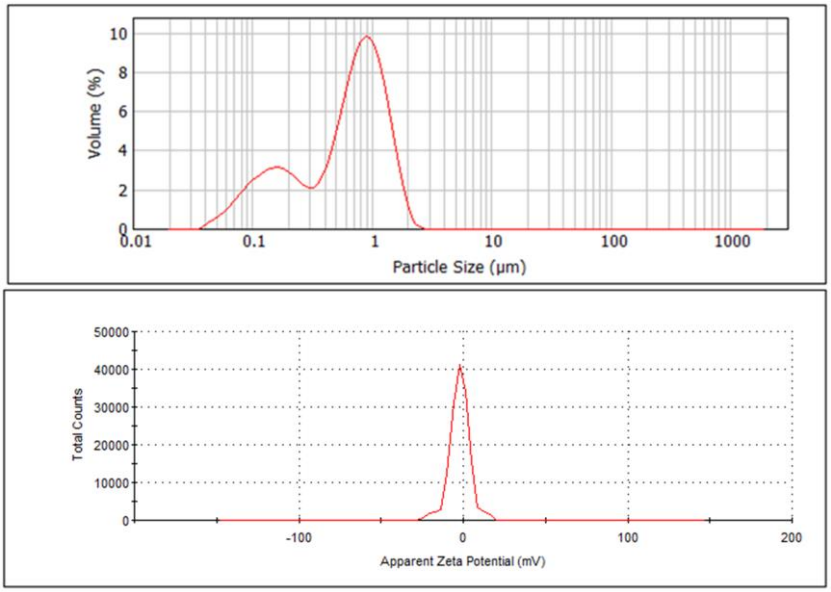

Figure 3. Particle size distribution (a) and zeta potential (b) of high viscosity liposomal formulation.

fluidity, two lipids with different transition temperatures were chosen here. Flexibility evaluation of prepared liposomes was performed using the method reported by Oluwatosin et al. that shows required time for extrusion of liposomes is in direct relation with their flexibility [16]. Our results revealed that extrusion of EPC-liposomes through 100 nm membrane filters was about twice faster than DSPC-liposomes in the same condition indicating higher flexibility for EPC-liposomes.

Changes in size distribution of liposomes with different flexibility are shown in Figure 4. Rigid liposomes showed great phase separation. Particle size ($D_{0.5}$) of these liposomes changed from 420 nm (at 15th hour) to 140 nm (at 26th hour) which is about 300% reduction ($P<0.05$). The changes in $D_{0.5}$ of flexible liposomes was much lower and only reduced by about 45 nm (about 10%); indicating that flexible formulation remains more homogenous over time.

Influencing forces in every particulate system, determines the behavior of suspending particles in that system. Sedimentation and Brownian motion are the most important factors that can affect the stagnating particles, which are discussed briefly below.

Sedimentation happens by the force of gravity and causes phase separation. This phenomenon is explained by Stoke's law. Based on this law, the rate of sedimentation is affected by the diameter of particles, gravity acceleration, medium viscosity of and the difference between the densities of particles and medium. Regarding this law, changes of size, density or viscosity can affect the rate of sedimentation. The Stoke's law also uses Anderson apparatus to measure the diameter

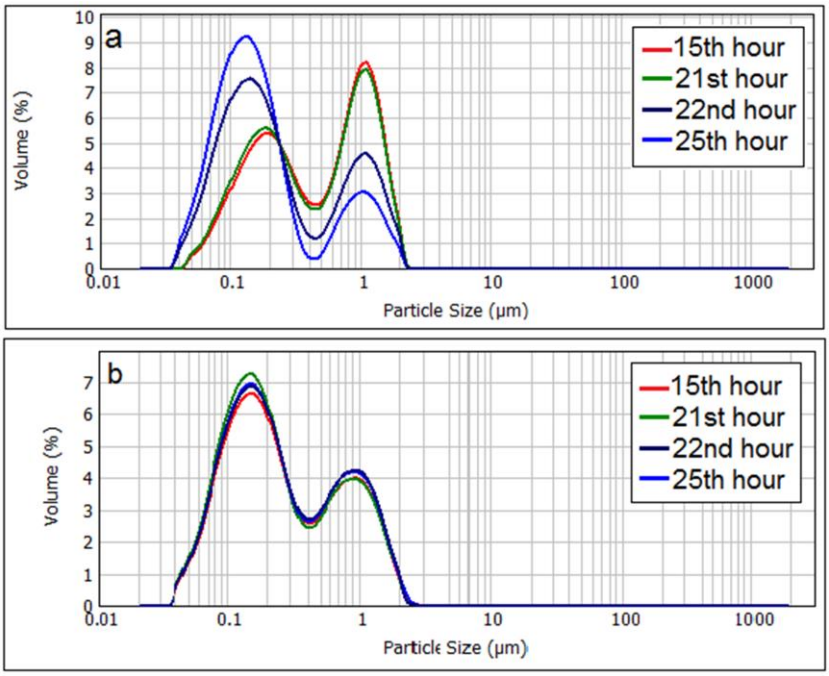

Figure 4. Effect of bilayer flexibility on size distribution of liposomal formulations. Samples were taken at different times (resembling different depth in the burette) (n=3).

of particles [12] which is close to the sedimentation method of the current study. Our model was developed to investigate the separation of nanoparticles under stagnation. This model can work with low volume in the same depth due to the narrower system.

Brownian motion is explained by Stoke's-Einstein law where, diffusion coefficient is affected by temperature, radius of particle and medium viscosity [17, 18]. Increase of Brownian motions increases the resistance of liposomes against sedimentation. Dominance of forces (gravitational and Brownian motion), defines the behavior of suspended particles.

As explained earlier our results show phase separation for rigid liposomes and, therefore it can be said that sedimentation was the dominant force that resulted in heterogeneity of system. On the other hand, preserved homogeneity of flexible liposomes shows that increasing the bilayer flexibility, changes the dominant force to Brownian motion and even large flexible liposomes did not sediment over time.

Brownian motion occurs in particles up to 5 µm and can prevent sedimentation in particle size of about 500 nm, which is claimed to be the Brownian motion border [12, 19], but our results show that changing the flexibility of liposomes could change this border. According to or results, sedimentation of rigid liposomes below the Brownian motion border was considerable over time and in higher depth, while flexible liposomes remained suspended; although there were particles as large as 2 µm in the system.

There is no data available about the effect of bilayer fluidity on separation of liposomes upon stagnation. Also, it has been reported that fluidity or flexibility of fibrous particles reduces their sedimentation coefficient due to the bending, class of symmetry, and changes in shape [20, 21]. It has also been discussed that the diffusivity and Brownian motions are possibly an influencing factor on the stability of suspensions containing flexible fibrous

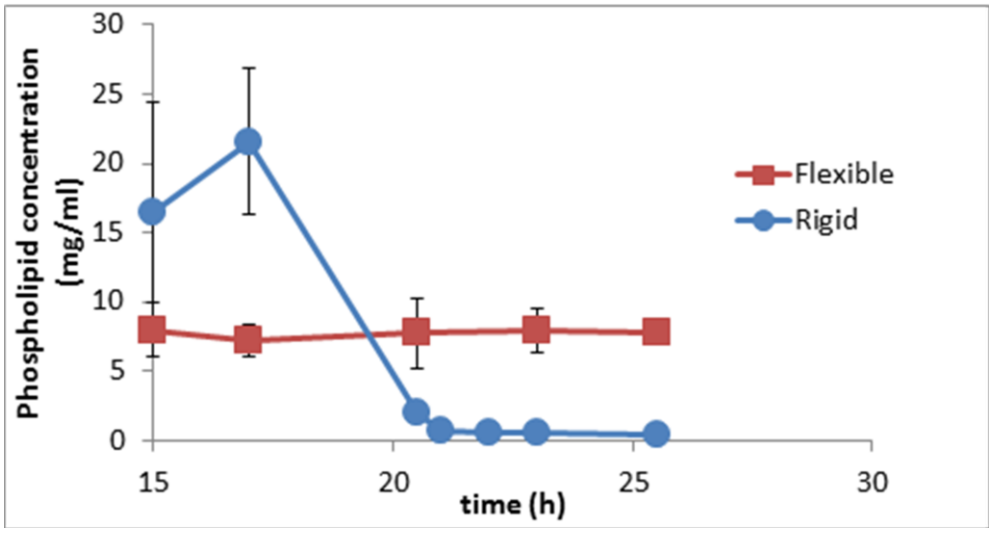

Figure 5. Effect of bilayer flexibility on phospholipid concentration, under gravity over time (n=3).

nanoparticles [22]. In agreement with the above mentioned discussions, our data show that increased flexibility of liposomes decreases phase separation and increases stability over time.

Changes in the phospholipid concentration during the experiment were also investigated as an indicator of liposomes' concentration (Figure 5). Statistical analysis showed that phospholipid concentration of rigid liposomes decreased by about 30 times over time and also was significantly different from other samples at each time point ($P<0.05$), while the phospholipid concentration of flexible liposomes remained unchanged ($P>0.05$) during the experiment (Figure 5). These results indicate that the flexible particles with different sizes do not tend to separate under gravity force, whereas the rigid particles tend to behave the opposite.

3.3. Effect of Viscosity

Viscosity can affect Brownian motion and gravitational forces in a medium. Therefor, it is important to know the effect of viscosity on the behavior of nanoparticles in convective flow. Water was replaced partly with propylene glycol to prepare a high viscosity liposomal formulation here. Viscosities of high and low viscosity liposomes with same lipid composition at shear rate of 60 s^{-1} was measured to be 45.2 and 3.4 cP respectively, using cone and plate viscometer (Brookfield,

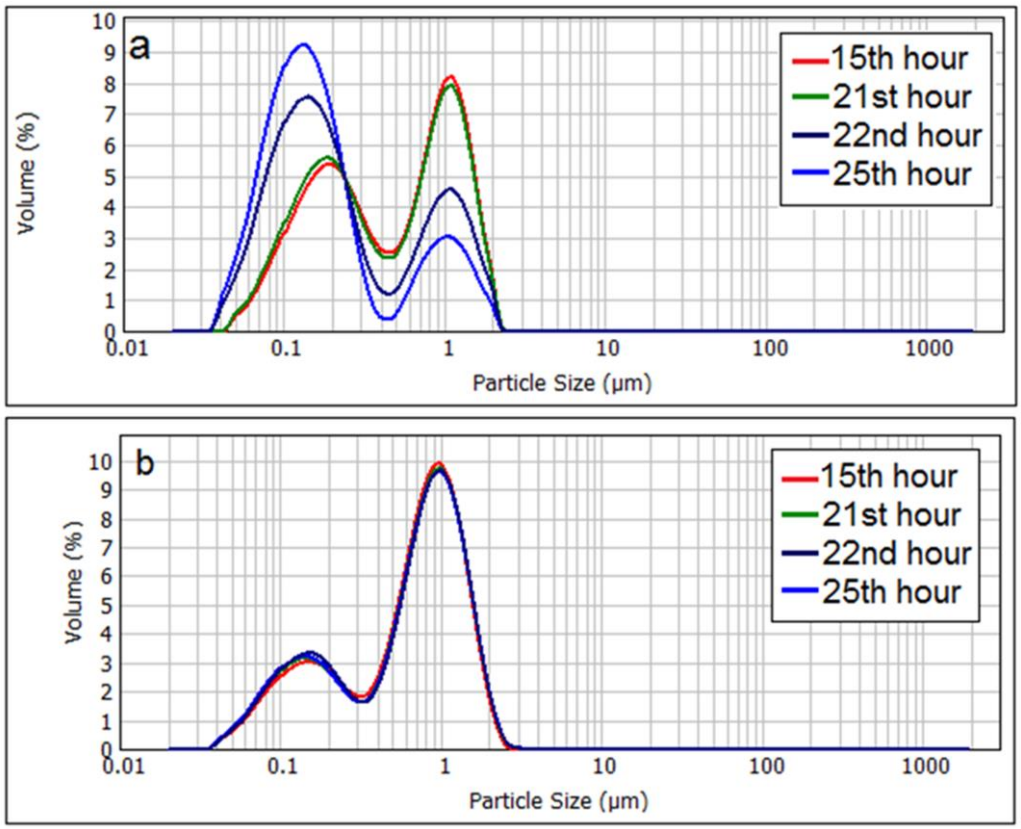

Figure 6. Effect of viscosity on size distribution of liposomal formulations. Samples were taken at different times (resembling different depth in the burette) (n=3).

USA). In phase separation studies, in low viscosity system, population of smaller liposomes grew significantly and $D_{0.5}$ of low viscosity liposomes reduced about 300% (to 140 nm at 26th hour). In high viscosity liposomal formulation, $D_{0.5}$ of liposomes did not change and this formulation remained homogenous over time (Figure 6).

aggregation of metal nanoparticles [23]. It has also been shown that viscosity of biofluids is effective on the movement of exosomes or microvesicles, biological interactions and fate of these lipid-based nano- and microparticle due to increased suspendability of lipid-based particles [24].

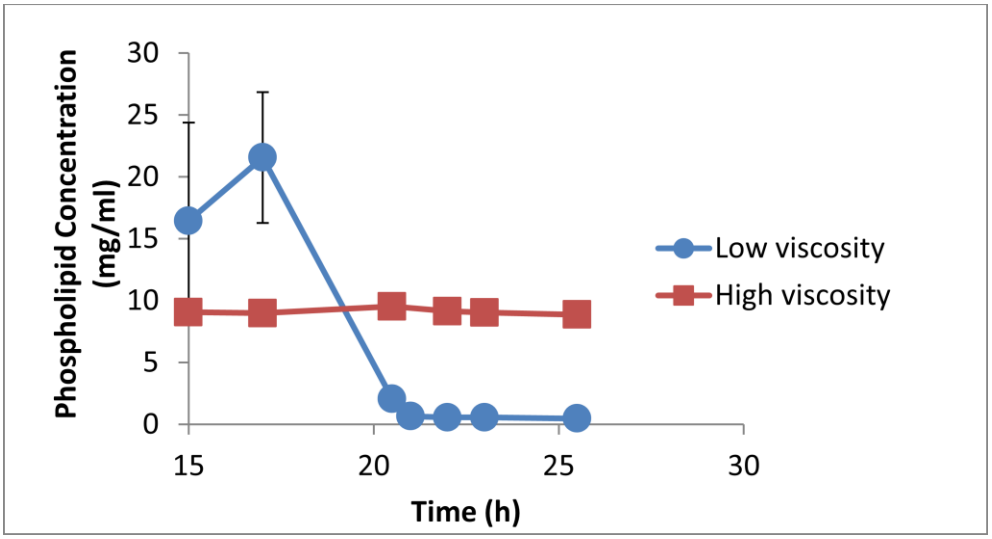

Figure 7. Effect of viscosity on phospholipid concentration, under gravity over time (n=3).

As discussed earlier, viscosity affects both sedimentation and Brownian motion of particles. According to our result, opposite behavior of liposomal formulations with different viscosities and preserved homogeneity of high viscosity liposomes show that viscosifying the system acted via preventing the sedimentation and phase separation and the system stayed homogenous in high viscosity formulation, despite of reduced motion of particles under the effect of Brownian motion, although there were particles as large as 2 µm in the system. Other studies also have reported that viscosity acts as an inhibiting factor on sedimentation and

Effect of viscosity on phospholipid concentration over time was also investigated. Phospholipid concentration in high viscosity liposomal formulation changed about 2%, which is negligible compared to more than 33 times reduction of phospholipid concentration (and also concentration of liposomes) in the low viscosity formulation (Figure 7).

4. Conclusion

Physicochemical properties of liposomes have been discussed widely to be of great importance in pre- and post-administrational fate of particles [25 - 28]. Condition or method of preparation, material and their source was

reported to have great influence on physicochemical properties of particles [29]. The present study investigated the effect of bilayer flexibility and medium viscosity on pre-administrational stagnation of liposomes. According to our results, both increasing the flexibility and viscosity have great influence on preserving the homogeneity of liposomes in suspending fluid.

There was great reduction of size (about 3 times) and concentration of phospholipids (about 33 times) in rigid and low viscosity liposomal formulation, which was also observed for particles smaller than the Brownian motion border. On the other hand, both high viscosity and flexible liposomes remained homogenous over time considering the particle size or phospholipid content even for liposomes as large as 2 µm. Therefore, it is important to note that liposomes with different physical characteristics (in this case, flexibility and viscosity) do not behave the same under gravity as a separating force.

It is not usual to control viscosity and flexibility of liposome preparation as an affecting parameter during formulation, while our results showed that these two parameters can highly affect the stability of liposomal preparations in stagnations even for short periods of time. The results of the present investigation also indicate that instability can occur in short time and can be very important in application of liposomes such as in infusion systems. The findings of the present investigation can be used in formulation of stable liposomes, alone or together with other methods of stabilization. Further studies are in progress in our laboratories to investigate the effect of convection flows on liposomal separations.

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