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Investigation on the stability of saquinavir loaded liposomes: Implication on stealth, release characteristics and cytotoxicity

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ABSTRACT

Although anti-retroviral therapy is the most efficient disease management strategy for HIV-AIDS, its applications are limited by several factors including the low bioavailability and first pass metabolism of the drugs. Nanocarriers such as liposomes have been developed to circumvent some of these problems. We report here preparation of novel liposome formulations for efficient delivery of anti-retroviral drugs to mammalian cells in culture. The liposomes were prepared and surface was modified using poly (ethylene glycol). Encapsulation efficiency of the anti-retroviral drug saquinavir was found to be approximately 33% and also exhibited sustained release of the drug. Although PEGylated liposomes were more stable in protein-supplemented media, had better colloidal stability and exhibited lesser sonochemical stability due to lower cavitation threshold. The cell viability studies using Jurkat T-cells revealed that the PEGylated liposomes loaded with saquinavir were less cytotoxic as compared to the non-PEGylated liposomes or free drug confirming the potential of the liposomes as a sustained drug-release system. The drug delivery potential of the liposomes loaded with Alexa flour 647 was evaluated using Jurkat T-cells and flow cytometry showing uptake upto 74%. Collectively, our data demonstrate efficient targeting of mammalian cells using novel liposome formulations with insignificant levels of cytotoxicity.

1. Introduction

Human immunodeficiency virus Type I (HIV-1) the causative agent for immunodeficiency in the human host manifests slow and progressive wasting disorders, neurodegeneration and death (Howard Temin, 1988). HIV-1 is morphologically similar to other lentiviruses that infect a range of other animals including feline immunodeficiency virus (cats), visna virus (sheep) and simian immunodeficiency virus (non-human primates). HIV-1 contains two copies of a single-stranded and positive sense RNA in the viral core. The viral RNA is transcribed through a complex process into double stranded DNA by the mediation of reverse transcriptase, and is integrated into the host genome by the mediation of another viral enzyme integrase (Fauci Anthony, 1988).

Anti retroviral therapy has been used successfully in the management of the viral infections as well as to control disease progression thereby improving the quality of life of the patient (Umesh and Narendra Jain, 2010). Depending on the chemical nature and the viral target, anti-retroviral medicines could be classified into several groups including nucleoside reverse transcriptase inhibitors (NRTIs), nucleotide reverse transcriptase inhibitors (NtRTIs), non-nucleotide reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), fusion inhibitors and integrase inhibitors (Lakshmi et al., 2010). Clinically, a combination of 3 or 4 individual drugs targeting at least two different components of the virus, technically referred to as highly active anti-retroviral therapy (HAART), is prescribed for treatment (Umesh and Narendra Jain, 2010).

Although HAART is the only disease management strategy available today, the therapeutic strategy suffers from severe limitations including drug resistance leading to poor clinical outcome (Amiji et al., 2006). Additionally, as the virus resides in the anatomical and cellular reservoirs of the human body that are typically inaccessible to drugs, majority of the anti-retroviral drugs and their combinations are inefficient in controlling the viral proliferation. Often the minimum therapeutic concentration of the drug required at the site of the viral localization is not achieved (Umesh and Narendra Jain, 2010). Furthermore, anti-retroviral drugs have low oral bioavailability due to first pass metabolism and undergo degradation in the

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gastrointestinal tract. The low half-life of the anti-retroviral drugs necessitates frequent administration of large doses, which in turn leads to low patient compliance (Li and Chan, 1999). Developing and optimizing the drug formulations remain a task of paramount importance for efficient targeting of anti-viral drugs and HIV disease management.

In the recent times, nanomedicine, which uses nano-scale materials for the diagnosis and therapy for diseases, has received a lot of attention. Nanocarriers have been able to effectively deliver drugs to target sites including the brain, a range of tumors and the liver by crossing biological barriers (Gupta et al., 2006; Covreur and Vautheir, 2006; Lockman et al., 2002). Polymer-based nanocarriers, non-polymer agents and biological carriers have been evaluated for the delivery of anti-retroviral drugs. Lamivudine-loaded fifth generation poly(propyleneimine) (PPI) and mannosylated-PPI (MPPI) dendrimers have been reported to significantly improve the anti-retroviral activity as compared to free drug (Dutta and Jain, 2007). Likewise, the targeting capability of efavirenz-loaded tuftsin-coated PPI dendrimers for HIV-infected macrophages, as compared to targeting the normal cells, was enhanced several fold (Dutta et al., 2007). Solid lipid nanoparticles (SLN) prepared from solid or semisolid fatty acids, stabilized with emulsifiers and co-emulsifiers have been used to encapsulate drugs such as AZT-palmitrate, trilaurin etc. (Heiati et al., 2008). Poly (butylcyanoacrylate), methylmethacrylate-sulfopropylmethacrylate nanoparticles and SLN have been used to study the transport of D4T, delaviridine and saquinavir across the blood-brain-barrier (Kuo and Su, 2007).

Liposomes contain a phospholipid bilayer that envelops an aqueous core. This unique property of liposomes enables the encapsulation of hydrophobic drug in the outer bilayer and a hydrophilic drug in the aqueous layer. A variety of liposomes cationic, anionic, sterically stabilized and immunoliposomes have been used for the delivery of anti-retroviral drugs such as zidovudine, didanosine and zalcitabine (Lanao et al., 2007). The surface of the liposomes can also be modified to improve circulation time, duration of drug release and enable efficient targeting. Furthermore, cholesterol has been incorporated into the liposomes to improve the rigidity resulting in the improved stability against serum lipoproteins and circulation time in the blood (Karlowsky and Zhanel, 1992; Vitas et al., 1996; Katragadda et al., 2000; Ashan et al., 2002).

We reported previously the delivery of a non-nucleoside reverse transcriptase inhibitor, nevirapine, using plain and modified liposomes (Lakshmi et al., 2010). In the present work we demonstrate efficient targeting of human T-cells by liposome containing a protease inhibitor, saquinavir. The aim of the present study was to load saquinavir in plain and modified liposomes and evaluate the release kinetics under various conditions along with the cell uptake and viability using Jurkat cells.

2. Materials and methods

2.1. Materials

Sodium dihydrogen phosphate and disodium hydrogen phosphate were purchased from Merck Chemicals, India. Egg phosphatidyl choline (EPC) was procured from Sigma–Aldrich, USA, DSPE-PEG was procured from Lipoid, Germany. Saquinavir was a kind gift from Hetero drugs, India. RPMI 1640 medium was procured from Sigma–Aldrich, USA and CellTiter 96® AQueous one solution was purchased from Promega, USA.

2.2. Preparation of saquinavir loaded liposomes

Liposomes were prepared using the thin film hydration technique. Briefly, 10 mg/mL of phospholipids in chloroform was taken in a clean container and was purged with nitrogen gas to remove the solvent. 1 mL of phosphate buffered saline (PBS, pH 7.4) containing saquinavir was added to the container and the mixture was warmed at 60 °C for 30 min with constant stirring. The solution was then extruded through polycarbonate membranes of 200 nm pore size using an extruder (Liposofast Basic, Avestin, Canada) for ten cycles to obtain extruded liposomes. The PEGylated liposomes were prepared essentially using the above described protocol with the exception that DSPE-PEG was added to the reaction along with Egg PC before purging with nitrogen.

2.3. Morphology of the liposomes

The morphology of the prepared liposomes was determined using transmission electron microscopy (TEM 2100 F, JEOL, Japan). The lyophilized samples were dispersed in 0.5 mL PBS and placed on a copper grid coated with carbon. The samples were left overnight for drying and then were imaged.

2.4. Determination of encapsulation efficiency

The extruded liposome samples were centrifuged at 15,000 rpm (Eppendorf 3340R, Germany) at 4 °C to pellet the drug-loaded liposomes (Okada et al., 1982). The supernatant was collected and the concentration of the drug left in the solution unencapsulated was measured at 239 nm using a UV–vis spectrophotometer (Lambda 25, PerkinElmer, USA). The absorbance was converted into drug concentration using a standard curve.

The encapsulation efficiency was calculated as:

$$\text{Encapsulation efficiency} = \frac{\text{Total drug} - \text{Unencapsulated drug}}{\text{Total drug}} \times 100$$

2.5. FTIR spectra

Infrared spectra were obtained in the transmission mode using a FTIR spectrometer (Spectrum 100, PerkinElmer, USA). KBr pellets of the plain liposomes or saquinavir loaded liposomes or free saquinavir were prepared using a hydraulic press. FT-IR analysis was performed between 4000 and 400 cm^{-1} at a resolution of 1 cm^{-1} averaging 10 scans.

2.6. Particle size analysis

The particle size of the plain and drug-loaded liposomes, which were PEGylated or non-PEGylated, was determined using the laser diffraction method (Microtrac Blue wave, Nikkiso, Japan) at room temperature. Five mL of the sample was dispersed in PBS. The mean size and size distribution of the liposomes was determined at a flow rate of 50%.

2.7. Zeta potential analysis

The zeta potential of the plain and drug-loaded liposomes, PEGylated and non-PEGylated, was determined using a Zeta sizer (Nano-ZS, Malvern, UK). One mL of the sample was dispersed in PBS and the mean zeta potential was recorded.

2.8. Thermal analysis

The thermal behavior of the plain and drug-loaded liposomes, PEGylated or non-PEGylated was evaluated using a differential scanning calorimeter (DSC, Q20, TA Instruments, USA). Two mg of the sample were placed in an aluminum pan along with the standard reference aluminum and the DSC was recorded between 10 °C and 90 °C at a scan rate of 10 °C/min for three cycles.

2.9. Release kinetics

The rate of saquinavir release from the liposomes was determined using the dialysis technique. The drug-loaded liposomes suspended in PBS were placed in a dialysis bag (Dialysis membrane 110, Hi Media, India), the bag was sealed at both the ends and immersed in 4 mL of 0.1 M PBS and dialysis was carried out at 37 °C against PBS, the PBS medium was removed after pre-determined time intervals for quantification of the drug released and replaced with the same quantity of fresh medium. To evaluate the influence of proteins on the stability of the drug-loaded liposomes, Dulbecco's Modified Eagle's Medium (DMEM, Hi Media, India) supplemented with 10% fetal bovine serum (FBS, HiMedia, India) was used for incubation instead of PBS medium. The effect of ultrasound on the release profile of the drug from the liposomes was studied in PBS (pH 7.4) as the release medium. Low frequency ultrasound (20 KHz) was applied using a sonicator probe. At predetermined time points, 4 mL of the release medium was withdrawn for analysis and replaced with 4 mL of fresh release medium. The amount of drug released was measured as absorbance at 239 nm using a UV–vis spectrophotometer (Lambda 25, PerkinElmer, USA). The absorbance was converted into percentage release using a standard curve as described above.

3. Colloidal stability

The colloidal stability of the liposome was determined based on the particle size measurement. The particle size was measured for both PEGylated and non-PEGylated drug-loaded and plain liposomes at various time points and also in the presence of protein at different condition of static and dynamic conditions along with zeta potential at 37 °C.

For static and dynamic conditions, 50 mg of the samples were added in a 500 mL standard flask filled with PBS supplemented with 10% serum protein at 37 °C. Fig. 1 depicts the schematic representation of the experimental set-up for static and dynamic conditions. To recreate dynamic circulation conditions, a peristaltic pump was used to circulate the release medium. The particle size and zeta potential of the samples were analyzed at 0, 2 and 6 h time points.

3.1. Evaluation of cytotoxicity in vitro

The cytotoxicity was evaluated in vitro for both PEGylated and non-PEGylated drug-loaded and plain liposomes in Jurkat T-cells (Origin of cells) using the MTS cell proliferation assay. Briefly, 2×10^4 cells in RPMI 1640 media were seeded in a 96-well flat bottomed tissue cultured plates. The cells were incubated at 37 °C in a 5% CO_2 incubator for 24 h. Appropriate concentrations of different liposomes suspended in PBS were added to the cells and the cells were incubated for 48 h. The free saquinavir dissolved in PBS served as positive control while untreated cells served as negative control. After incubation, the cells were washed with PBS and MTS assay was performed as per the manufacturer's instructions (Anuradha et al., 2011).

3.2. Evaluation of in vitro cytotoxicity and liposome delivery using flow cytometry

Jurkat T-cells, 0.5×10^5 cells per assay, were incubated in 200 μL of plain RPMI containing plain PEGylated liposome (PL) or saquinavir (SQV)-loaded PEGylated liposome, normalized for 300 or 600 μg of total lipid. The cells were incubated at 37 °C in 5% CO_2 incubator for 30 min and washed twice in 1X PBS. Cell death was determined by flow cytometry immediately following drug delivery or after resting cells for 12 h in RPMI medium supplemented with 10% fetal calf serum, glutamine and antibiotics. Cells were stained with Live/Dead Fixable Far Red Stain Kit (Invitrogen) for 30 min in dark as per manufacturer's instructions. Cells were washed twice in PBS and fixed in 400 μL of 1% paraformaldehyde before acquisition using FACS Calibur flow cytometer. Data were analyzed using BD CellQuest Pro software for Windows and compensation was performed using single-stained controls and gates were set using appropriate controls. All the experiments for cell viability have been performed in triplicate wells. Appropriate controls that were treated with comparable concentration of soluble SQV or no treatment (NT) were included. An unpaired two-tailed *t*-test was performed using Graphpad Prism 5 software to determine the significance of differences in the magnitude of cell death between different groups. Cells were treated with Alexa fluor 647 loaded PEGylated liposomes as per the above mentioned protocol to determine cell targeting efficiency.

3.3. Evaluation of liposome delivery using confocal microscopy

Jurkat T-cells in 200 μL of plain RPMI, 0.5×10^5 cells per assay, were treated with 300 μg of plain liposome (PL) or FITC-loaded PEGylated liposome. The cells were incubated in an incubator at 37 °C in the presence of 5% CO_2 for 30 min. Cells were washed twice in PBS, resuspended in 200 μL of PBS supplemented with 5 μg of Hoechst 33,258 dye and incubated at room temperature for 30 min. Cells were washed once with PBS, mounted in 70% glycerol for imaging analysis. Imaging was performed on Carl Zeiss LSM510 Meta confocal laser scanning microscope using a Plan Apochromat X63/1.4-oil immersion objective and analyzed using the LSM image examiner software (Carl Zeiss, Inc.).

3.4. Statistical analysis

All data are represented as mean ± standard deviation ($n = 3$). The analysis of variances was calculated using two-way ANOVA and Tukey test was performed to test level of significance for cell culture assay.

4. Results and discussion

4.1. Morphological analysis of the liposomes

The spherical morphology of the saquinavir-loaded liposomes is evident in the transmission electron micrographs (Fig. 2). The morphology and the size of the liposomes do not seem to undergo drastic changes suggesting that the bulk of the drug is localized to the core of the liposomes.

4.2. Encapsulation efficiency

The encapsulation efficiencies of saquinavir were approximately 31.2 ± 1.02% and 32.21 ± 2.86%, for non-PEGylated and PEGylated liposomes, respectively. Encapsulation efficiencies of saquinavir in non-PEGylated and PEGylated liposomes did not differ significantly from each other. Similar observations were observed in the case of topotecan encapsulated in non-PEGylated and PEGylated liposomes by Yan-le et al. (Yan-le et al., 2005). This result is in corroboration with our inference drawn from the particle size determination and zeta potential analysis that the drug predominantly localizes to the aqueous core of the liposomes. Since the volume of the aqueous core remains constant in unilamellar vesicles of the same size range, the encapsulation percentages of the drugs are not expected to change if the cargo is localized to the aqueous core as depicted schematically.

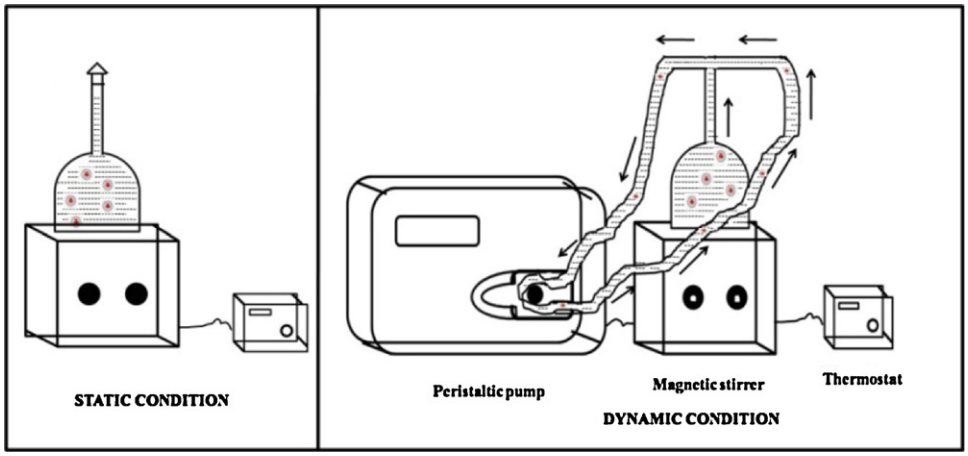

Fig. 1. Experimental setup depicting [A] the static and [B] dynamic conditions used the release of saquinavir from the liposomes.

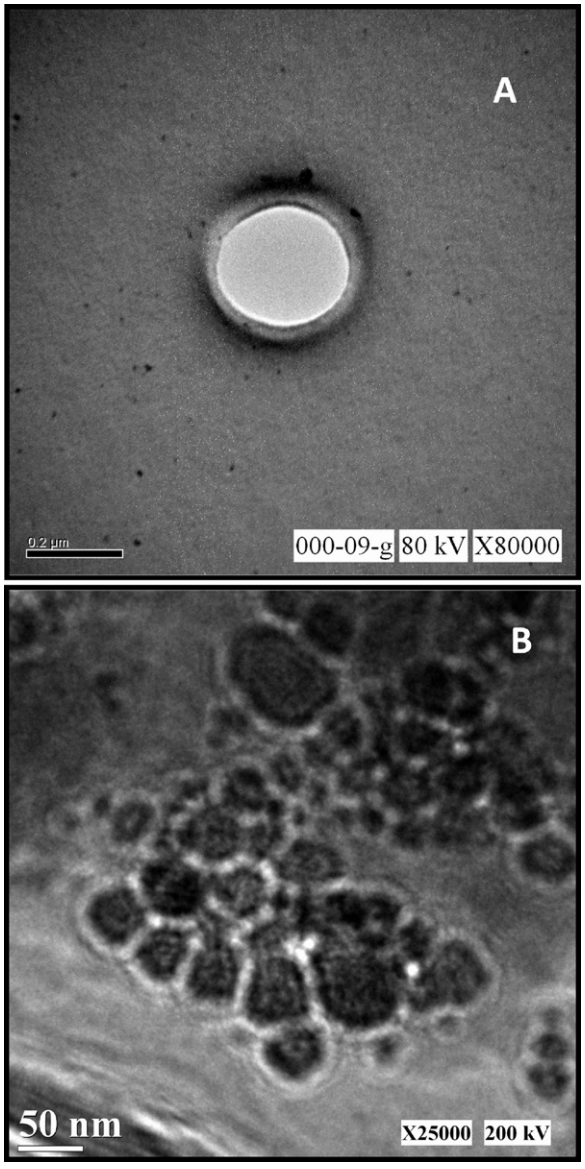

Fig. 2. Transmission electron micrograph of (A) plain liposome (B) saquinavir-loaded liposomes.

4.3. FTIR spectroscopy

We examined the emission spectra of free saquinavir, plain liposomes and saquinavir-loaded liposomes (Fig. 3). The FTIR spectrum of the drug loaded liposome showed the characteristic peaks for saquinavir at 3353 cm^{-1} due to amide —N—H— stretch and hydroxyl stretch and 1673 cm^{-1} due to the amide carbonyl, and 1550 cm^{-1} due to —N—H bend and 699 cm^{-1} due to out of plane aromatic bend. These peaks were absent from the plain liposome thereby comprising the presence of saquinavir in the liposome. The FTIR spectrum of the drug loaded liposome also shows distinct band at 1737 cm^{-1} due to ester carbonyl groups and 1099 cm^{-1} due to —C—O— of ester which are also found in the spectrum for plain liposomes.

4.4. Particle size

Table 1 illustrates the values for the zeta potential and particle size obtained for plain and drug-loaded liposomes with or without PEG. The mean particle size of the plain and saquinavir-loaded liposomes does not show a significant difference either in the presence or absence of PEGylated lipids implying that the PEG chains makes no contribution to the particle size. The

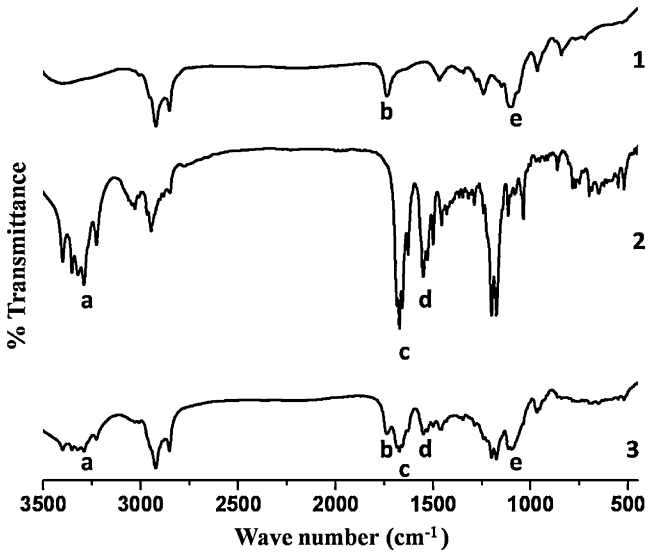

Fig. 3. FTIR spectra of [1] plain liposomes, [2] free saquinavir and [3] saquinavir-loaded liposomes (a = 3353 cm^{-1}, b = 1737 cm^{-1}, c = 1673 cm^{-1}, d = 1550 cm^{-1}, e = 1099 cm^{-1}).

Table 1
Zeta potential and mean particle size properties of the PEGylated and non-PEGylated, plain and drug-loaded liposomes.

	Egg phosphatidyl choline (10:0)		Egg phosphatidyl choline:DSPE-PEG (9:1)	
	Size (nm)	Zeta potential	Size (nm)	Zeta potential
Plain liposomes	173.00 ± 4.35	−37.33 ± 1.58	175.00 ± 10.10	−46.06 ± 1.35
Saquinavir loaded liposomes	174.30 ± 13.30	−28.60 ± 1.28	176.60 ± 6.80	−35.50 ± 1.66

presence of saquinavir did not appreciably alter the particle size indicating that most of the drug is probably localized to the aqueous core of the liposome which is unlikely to modify the particle size. Saquinavir being partially soluble in water and having polar groups is likely to localize to the lipid-electrolyte interface found in the core. Similar observations have been made previously by Vono et al., 2010 for liposomes loaded with gemcitabine, a hydrophilic anti-cancer drug (Margherita et al., 2010).

4.5. Zeta potential

The zeta potential measurements of the plain liposomes show a pronounced shift towards negative values when PEGylated. This shift may be attributed to the hydrophilicity and fast chain dynamics of PEG. 'Fast chain dynamics' refers to the fast movement of the PEG chains on the surface of the carrier. This phenomenon has been reported to hinder adsorption on the surface of carriers (Tirosh et al., 1998) as well as shift the zeta potential towards negative values (Vono et al., 2010). The PEG chains are able to effectively mask the negatively charged phosphate groups in the egg phosphatidyl choline forming the liposome from being neutralized by the positively charged ions from the medium. Additionally, the DSPE-PEG chains also contribute to the negative zeta potential through their anionic phosphate groups. The negative zeta potential of the liposomes is shifted to slightly positive values in the case of saquinavir-loaded liposomes both in the presence as well as absence of PEG. The magnitude of change remains the same both in PEGylated liposomes and non-PEGylated liposomes (22.93% and 23.41% respectively) implying that this shift is solely due to the interaction of the polar groups of the drug with the negatively charged groups in the phospholipids forming the liposome. Moreover, a small amount of the drug may also be found at the lipid-electrolyte interface on the surface of the liposome (Paola et al., 2007).

4.6. DSC thermograms

The gel-liquid crystalline phase transition is the temperature below which the lipid bilayer exhibits an ordered arrangement and above this temperature, the acyl chains have less order and hence exhibit enhanced permeability. The gel-liquid crystalline phase-transition temperatures of liposomes do not show a significant difference between blank and saquinavir-loaded liposomes (Table 2). This observation confirms our earlier conclusion that the drug is present inside the inner aqueous core of the liposome (ElMaghraby et al., 2005). The PEGylated liposomes exhibit a higher phase transition temperature than the conventional liposomes due to a change in the Gibbs free energy of the gel and liquid crystalline phases of the liposomes (Kaname et al., 2006).

Table 2
Phase-transition temperatures of non- PEGylated and PEGylated liposomes.

	Phase-transition temperature (°C)	
	Egg phosphatidyl choline (10:0)	Egg phosphatidyl choline:DSPE-PEG (9:1)
Plain liposomes	37.27	49.52
Saquinavir loaded liposomes	36.38	48.33

4.7. In vitro release

The in vitro release profile of saquinavir from the non-PEGylated and PEGylated liposomes at 37 °C in PBS has been presented (Fig. 4). The profile indicates that PEGylated liposomes exhibit a sustained release of the saquinavir as compared to non-PEGylated liposomes. An initial burst release was observed in both non-PEGylated and PEGylated liposomes. However the burst release was more pronounced in non-PEGylated liposomes as compared to PEGylated liposomes. Nearly 32% of saquinavir was released from the non-PEGylated liposomes within 30 min and most of the drug was released in 20 h. On the other hand, only 14% of saquinavir was released from the PEGylated liposome in 30 min and a complete drug release required 50 h of incubation (Fig. 4).

All in vitro release data has been modeled mathematically using the Higuchi equation which is plotted between the cumulative concentration of the drug and the square root of time. The Higuchi constant K_H was calculated from the plot by determining the slope of the linear line passing through the origin. Fig. 5 shows the Higuchi plot for the various drug release conditions i.e. in PBS, DMEM medium supplemented with 10% FBS and ultrasonication. The equation based on the Higuchi model predicts the cumulative drug release (%) versus (time)$^{0.5}$ data for non PEGylated liposomes in PBS with a correlation coefficient of 0.975 for 28 time points and a standard deviation of 4.09%, PEGylated liposomes in PBS show a correlation coefficient of 0.965 for only the initial 4 time points with a standard deviation of 4.15%. The Higuchi constant for PEGylated liposomes in PBS medium is 13.28 while for the non-PEGylated liposomes, it is 39.40 in the initial phase. The higher value of K_H is indicative of a greater release. Thus it is obvious that the release rates in PBS for PEGylated liposomes are significantly lower when compared to non-PEGylated liposomes. While PEGylated liposomes show excellent conformity to the Higuchi kinetics indicating a diffusion controlled release, the non-PEGylated liposomes show a pronounced shift from the Higuchi kinetics beyond 2 h. This indicates that the burst release of nearly 50% of drug in the initial phase alone is driven by diffusion while beyond 2 h other factors contribute to the deviation. In case of DMEM supplemented with 10% FBS, non-PEGylated liposomes exhibit a correlation coefficient of 0.957 for 8 time points with a standard deviation of 5.79 and PEGylated liposomes show a correlation coefficient of 0.957 for 5 time points with a standard deviation of 6.68. Both carriers exhibit good correlation coefficients during the entire period of release with the Higuchi equation indicating that the release is diffusion controlled. The K_H values for PEGylated liposomes is 39.69 while for the non-PEGylated liposomes, it is 58.93. This represents a three-fold increase in the case of PEGylated liposomes in the protein supplemented medium when compared with the release in PBS. This indicates that presence of protein tends to destabilize the lipid bilayer. However, the non-PEGylated liposomes show much higher release when compared to their PEGylated counterparts indicating that the PEG chains tend to retard the protein interaction with the bilayer components. In case of ultrasonication, non PEGylated liposomes show a correlation coefficient of 0.983 for 9 time points with a standard deviation of 3.31 and PEGylated liposome shows

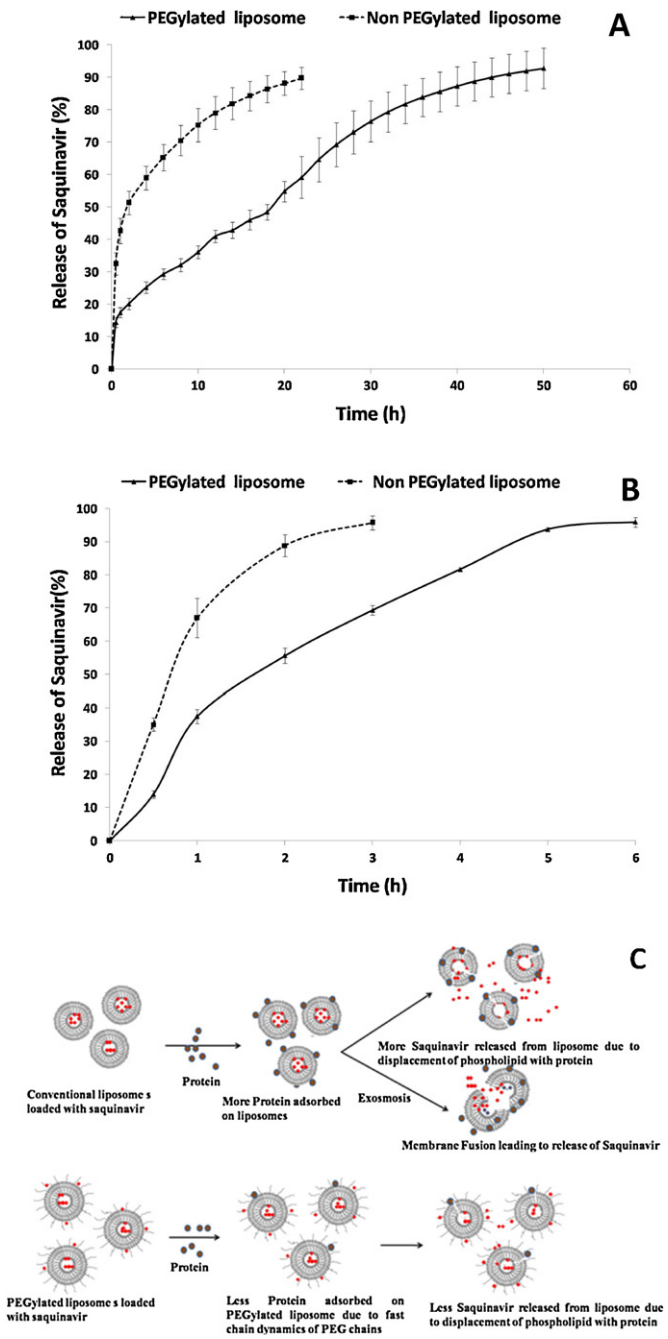

Fig. 4. Drug release profiles of saquinavir from non-PEGylated and PEGylated liposomes in presence of (A) PBS, (B) serum protein and (C) schematic representation of liposome fluidization in a protein containing medium.

a correlation coefficient of 0.960 for 6 time points with a standard deviation of 5.28. Both systems conform to the Higuchi diffusion model as is evident from the good correlation coefficients. The K_H values for PEGylated and non-PEGylated liposomes when subjected to ultrasonication were found to be 66.00 and 50.00 respectively. This represents a five-fold increase in the case of PEGylated liposomes when subjected to ultrasonication as compared to its release kinetics in PBS. Interestingly, the non-PEGylated liposomes show relatively lesser drug release under the same conditions when compared with the PEGylated carriers. This highlights the role of the PEG chains in lowering the cavitation threshold of liposomes leading to extremely high rates of drug release.

Release of a drug from the liposome is mainly diffusion-controlled and the fluidity or rigidity of the liposomal membrane lipids also influences the rate of diffusion of the drug. The state of the membrane in turn is influenced by temperature, pH, presence of fluidizing agents, zeta potential and nature of the phospholipids. The presence of PEG on the liposome surface retards the diffusion of saquinavir from the interior owing to its fast moving hydrophilic chains. Furthermore, the high negative zeta potential of the PEGylated liposomes tends to increase liposome stability, and reduce membrane fusion and fluidity thus possibly contributing to reduced drug diffusion out of the liposome (Jie et al., 2009).

The effect of the presence of proteins on the kinetics of drug release from non-PEGylated and PEGylated liposomes was determined by monitoring the release of saquinavir in protein containing DMEM medium. Blood is a complex mixture of molecules, minerals, vitamins and proteins. The stability of the liposome can be affected in the presence of proteins. As the DMEM-FBS combination provides a milieu similar to that available in blood and is used as the medium for cell culture studies, experiments were carried using DMEM-FBS. Similar studies have been reported in literature by (Tove et al., 2010a,b). The release of saquinavir for non-PEGylated and PEGylated liposomes showed a burst release of 67% and 46%, respectively, after 1 h (Fig. 6) which was 3- and 2-fold higher as compared to drug release in PBS for non-PEGylated and PEGylated liposomes, respectively. It is possible that protein adsorption to liposome surface may cause displacement of the phospholipids from the bilayer, fluidization of the liposomal bilayer, thereby leading to accelerated rates of the drug release from the liposomes. Furthermore, protein adsorption may also lead to increased osmotic stress across the liposomal bilayer membrane due to displaced or neutralized charges in the hydration layer surrounding the liposome. As a consequence, the aqueous layer containing the drug from the core may be expelled resulting in the exosmosis and fusion of the liposomal membranes. A similar phenomenon, however, is not expected in the case of PEGylated liposomes as the presence of the fast moving hydrophilic chains of PEG on the liposome surface is likely to retard protein adsorption and delay neutralization of the surface charges, displacement of phospholipids and subsequent fluidization of liposome (Hee et al., 2006).

Drug release kinetics from the liposomes was nearly twice as faster when subjected to ultra sonication (Fig. 6) as compared to that in the presence of protein (Fig. 4b). For instance, with non-PEGylated liposomes, 100% of the drug was released within 2 and 3.5 h with ultrasound and presence of proteins, respectively. Application of ultrasound produces air bubbles, which expand and compress during the rarefaction and compression phases of the sound waves. At a particular point known as the 'cavitation threshold', the bubbles implode producing enormous energy locally and transiently (Jagannathan et al., 2003). Greater number of bubbles is expected to cause enhanced energy release and increased free radical production resulting in the faster drug release profile. PEG is hydrophilic and hence attracts water resulting in creation of bubbles on application of ultrasound. The expected mechanism of drug release from liposomes following the application of ultrasound is depicted schematically (Fig. 6b).

4.8. Colloidal stability

The colloidal stability of liposomes was examined in the presence of proteins as this quality of the liposomes is expected to influence the extent of immune recognition, residence time, cellular uptake and drug release kinetics in vivo. The flow rate of the blood in vivo is an additional and essential parameter that could influence the fate of the liposomes including immune recognition, cellular uptake and drug release. Proteins tend to adsorb to

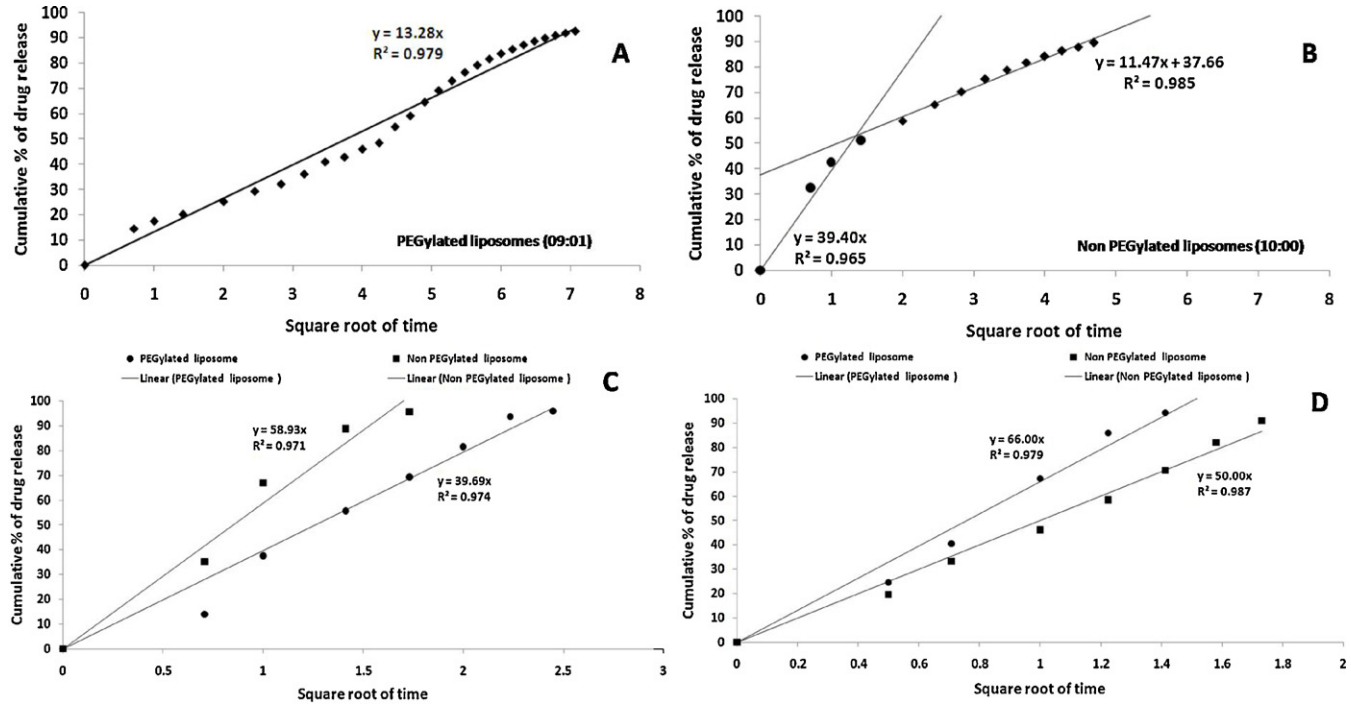

Fig. 5. Mathematical model of in vitro release. (A) PBS - PEGylated liposome, (B) PBS - non PEGylated liposome, (C) FBS, (D) ultrasonication.

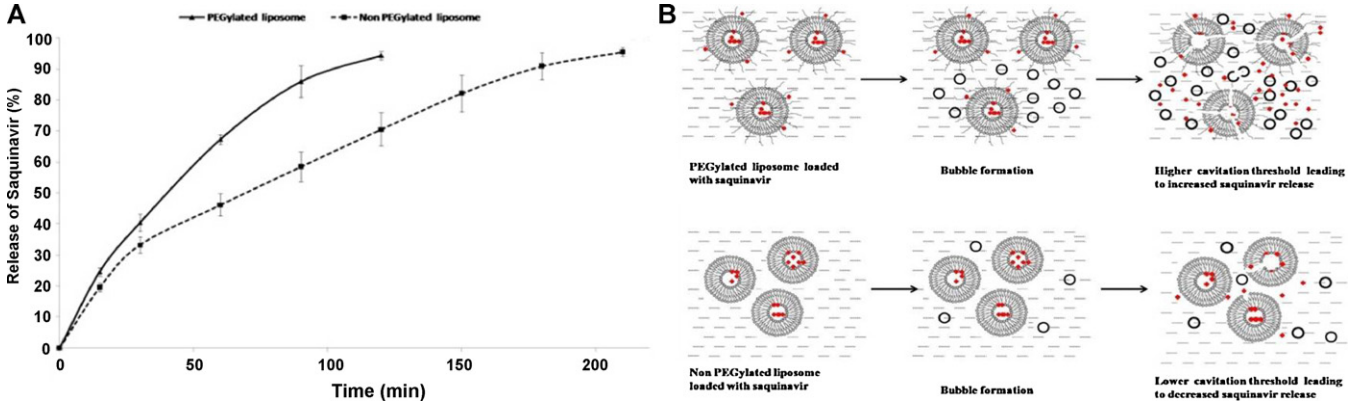

Fig. 6. Drug release profile from liposomes suspended in PBS and subjected to (A) ultrasonication, (B) schematic depiction of the expected mechanism of drug release from liposome following the application of ultrasound.

liposome surface thus altering the zeta potential of the liposomes and their size distribution.

The zeta potential of saquinavir-loaded non-PEGylated and PEGylated liposomes becomes progressively negative as a function of time in the presence of protein under both static and dynamic conditions (Gabriele and Gregor, 1993). The PEGylated liposomes exhibit significantly lesser magnitude of change in the zeta potential as compared to the non-PEGylated liposomes under static as well as dynamic conditions. The zeta potential values obtained at various time points for non-PEGylated and PEGylated liposomes under static and dynamic conditions have been summarized (Table 3).

The changes in the zeta potential values of liposomes may be attributed to the masking or neutralization of the surface charges by protein adsorption to the liposome surface. Liposomes are surrounded by a hydration layer containing charged ions that may be displaced by the protein adsorption and also contributing to the changes in the zeta potential (Sabin et al., 2006). PEGylated liposome and non-PEGylated liposome responded alike in both static and dynamic condition with reduction in negative zeta potential of 25% and 79% respectively after 6 h of incubation in protein medium. These observations indicate that both PEGylation and dynamic flow of the medium containing proteins provide similar environment for adsorption. In dynamic flow conditions, the constant movement

Table 3
Zeta potential in the presence of protein under static and dynamic conditions.

Time (hours)	Zeta potential (mV)	
	Non-PEGylated liposome	PEGylated liposome
	Static condition	Static condition
0	−28.9 ± 1.2	−35.5 ± 1.6
2	−19.1 ± 1.3	−31.1 ± 1.1
6	−4.2 ± 1.2	−20.5 ± 0.4
	Dynamic condition	Dynamic condition
0	−31.6 ± 1.8	−36.5 ± 2.0
2	−25.9 ± 1.7	−32.3 ± 1.3
6	−20.7 ± 2.2	−27.4 ± 0.5

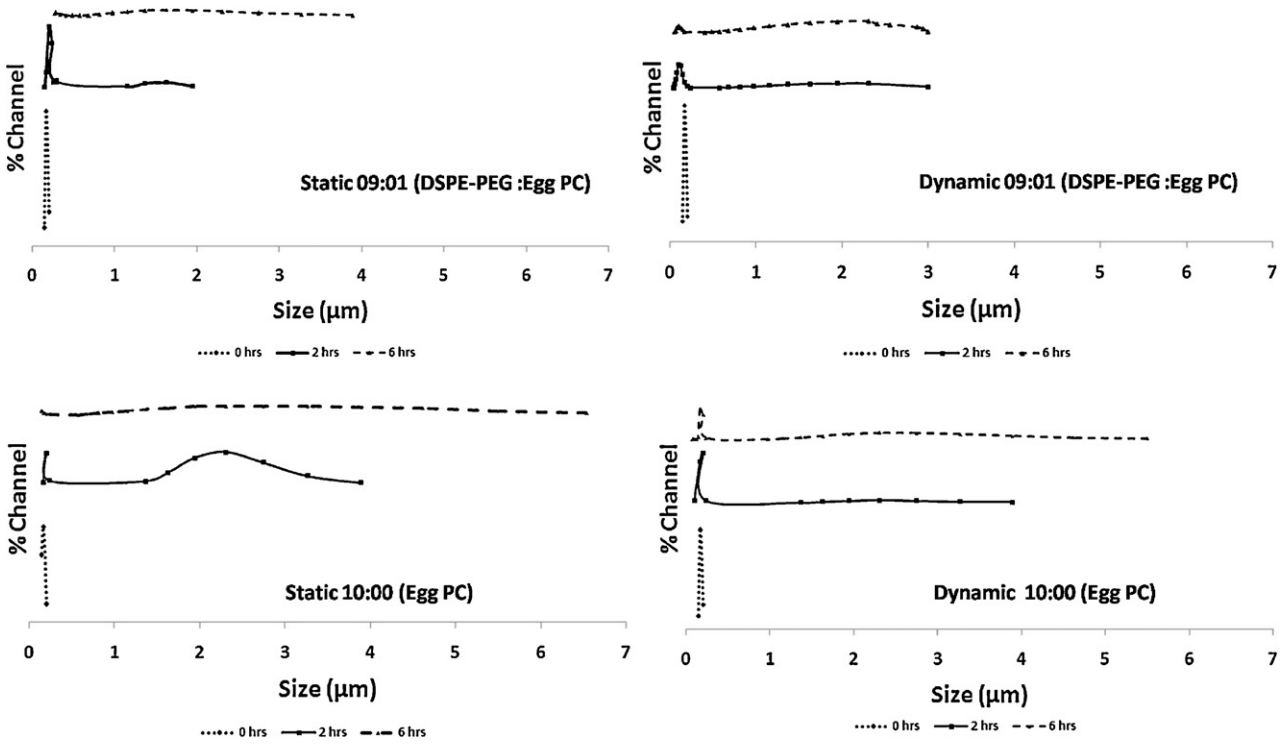

Fig. 7. Colloidal stability of the liposomes under static and dynamic conditions.

of proteins reduces the time of interaction with the liposome surface thereby lowering the magnitude of zeta potential change. The dynamic flow and PEGylation of the liposomes collectively are expected to offer maximum resistance to protein adsorption and result in least changes in the zeta potential.

The influence of the presence of protein on the particle size of non-PEGylated and PEGylated liposomes under static and dynamic conditions has been depicted as a function of time (Fig. 7). With time, micron- as well as nano-sized liposome fractions begin to appear. The increase in the micron-sized fractions is highest for non-PEGylated liposomes under static conditions while both dynamic flow conditions as well as PEGylated liposomes display slow increase in the micron-sized population implying better colloidal stability. The adsorption of the proteins disrupt the hydrated layer, masking or neutralizing the surface charges thereby promoting liposome aggregation and increasing the particle size (Jubo et al., 2006). The fast moving PEG chains on the surface have a higher volume of hydrated layer owning to the its hydrophilicity. These factors contribute to increased colloidal stability through both steric repulsion by PEG chains as well as electrostatic repulsion by the ions in the hydrated layer thus retarding the increase in the particle size. Dynamic flow conditions keep the proteins away from the liposome surface thus contributing to improved colloidal stability.

4.9. In vitro cytotoxicity study using MTS assay

The cytotoxic property of saquinavir loaded to non-PEGylated or PEGylated liposomes was evaluated using Jurkat cells and the MTS assay. These results were compared with those obtained using the free drug (Fig. 8).

The drug exhibited a dose-dependent decrease in cell viability in all the forms to a small extent from 54 to 43% for plain drug, 62 to 49% for non PEGylated liposome and 79–70% at 10–50 μg. The magnitude of decrease is not drastic and hence the scale has been magnified to show the dose dependency in Fig. 8. However, free saquinavir exhibited higher levels of cytotoxicity as compared to liposome-loaded drug at equivalent concentration. This observation may be attributed to the steric hindrance offered by the PEG chains on the liposome surface, which retards the diffusion of the drug and also due to lower cell uptake due to its surface hydrophilicity.

4.10. In vitro cytotoxicity and cellular uptake using flow cytometer

To determine the cell targeting efficiency of the liposomes, we prepared PEGylated liposomes loaded with a fluorescent molecule Alexa Fluor 647 or as plain liposomes without the dye. Jurkat T-cells were incubated with plain or dye-loaded liposomes, at two different lipid concentrations −300 or 600 μg of total lipid/assay, for 30 min. Cells were fixed in 1% paraformaldehyde and the fraction positive for fluorescence was determined using FACS Calibur. Approximately 55 and 74% of cells were positive for fluorescence under these experimental conditions at the two lipid concentrations, respectively, confirming efficient cargo delivery (Fig. 9A). The targeting efficiencies are highly reproducible. Importantly, using a commercial kit (Live/Dead Fixable Far Red Stain Kit, Invitrogen) and flow cytometry, we confirmed that cell death was quite low as compared to the untreated control cells ranging from 2.6 to 10.4% suggesting that the lipid formulations are not cytotoxic under the assay conditions (Fig. 9B). The rate of cell death remained the same at low levels regardless of additional resting period provided to the cells for recovery from a possible injury (Data not shown). Confocal imaging of the cells following FITC-loaded PEGylated liposome delivery to Jurkat cells showed uniform distribution of the fluorescent dye to cytoplasm and nucleus thus confirming efficient

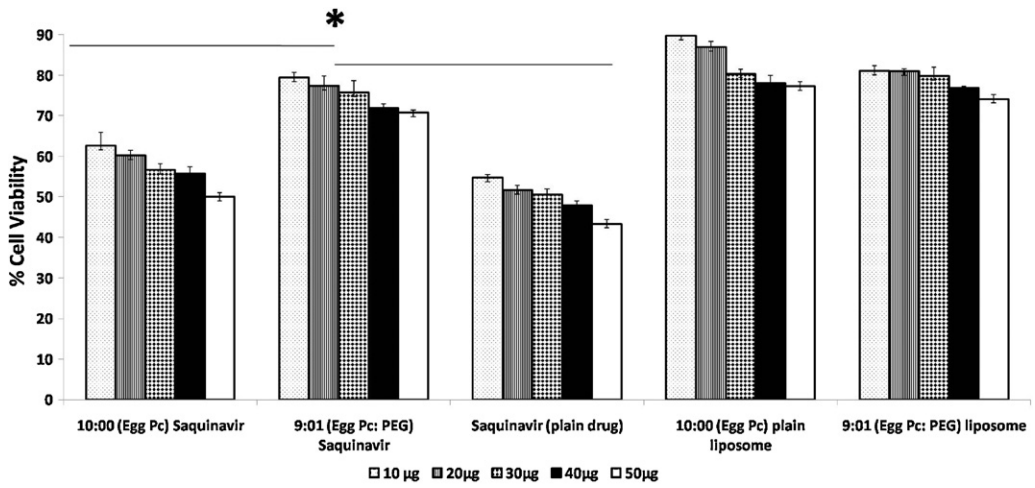

Fig. 8. Cell viability of free and liposome-loaded saquinavir (*–$p < 0.05$). Statistical comparison was made using ANOVA.

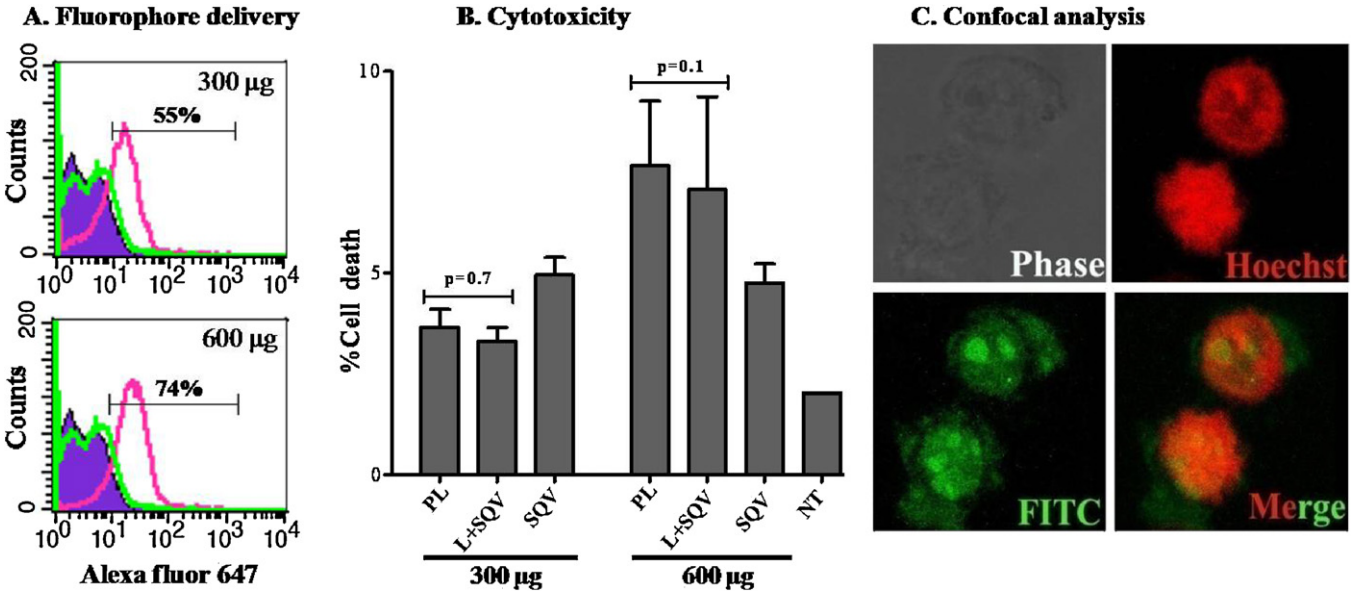

Fig. 9. Cell targeting efficiency of the PEGylated liposomes in Jurkat T-cells. (A) Jurkat cells were treated with Alexa fluor 647 loaded PEGylated liposomes to determine cell targeting efficiency. The blue histogram represents unstained cells, green color control lipid delivery without the fluorophore and the red color fluorophore-loaded liposome delivery. The percentage of cells stained for the fluorophore depicted. (B) Jurkat T-cells were treated for 30 min with plain liposome (PL) or liposome (L) + saquinavir (SQV) normalized for 300 or 600 µg of total lipid. The magnitude of cell death immediately following drug delivery was determined by using a commercial kit and flow cytometry. Appropriate controls where cells were treated with comparable concentration of soluble SQV or no treatment (NT) were included. An unpaired two-tailed test was performed to determine the significance of differences in the magnitude of cell death between PL and L + SQV groups. The data are from one of the three representative experiments. (C) Confocal images showing delivery of FITC-loaded liposomes to Jurkat cells. The FITC-loaded liposomes are chemically identical to those used in panel 'A' above. Nuclear staining by Hoechst has been color coded to red and the green color represents FITC. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

delivery of the cargo to the cells and ruling out the possibility that the liposomes only adhered to the cell membrane without undergoing a fusion event (Fig. 9C).

The low level cell death observed with 'saquinavir only' treatment of the cells in this assay, as opposed to the MTS assay above (Fig. 8) could be the consequence of reduced exposure time of the cells to the drug, 30 min as opposed to 24 h. Interestingly, we found that non-PEGylated liposomes are superior in drug delivery properties with the targeting efficiencies reaching as high as 90% magnitude under identical experimental conditions (data not presented). The non-PEGylated liposomes, however, also demonstrated higher levels of cell death thus offsetting the advantage. Similar results have been reported previously for Paclitaxel, an anticancer drug (Yang et al., 2007).

5. Conclusion

We demonstrated preparation of novel PEGylated liposomes using thin film hydration technology. PEGylated liposomes showed higher stability in protein supplemented media. Drugs and fluorescent dyes encapsulated into the liposomes by passive loading were released at sustained kinetics up to 50 h. The encapsulation efficiencies of the PEGylated liposomes are comparable to the non-PEGylated liposomes. In addition, the PEGylated liposome exhibits better colloidal stability in presence of protein when compared with the non PEGylated liposomes. More sustained release of the drug from the carrier is observed in PEGylated liposomes when compared with the non-PEGylated liposomes. Our data collectively prove a safe and efficient strategy for sustained drug

delivery to target cells mediated by PEGylated liposomes. Importantly, liposome-mediated drug delivery might offer the benefit of drug stability, reduced dose frequency and low cytotoxicity.

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