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Research Article

Efavirenz Loaded Nanostructured Lipid Carriers for Efficient and Prolonged Viral Inhibition in HIV-Infected Macrophages

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Abstract

Background: The clinical outcome of anti-HIV therapy is poor due to the inherent fallouts of anti-HIV therapy. It is further worsened due to the presence of viral reservoirs in immune cells like the macrophages. An ideal anti-HIV therapy must reach, deliver the drug and exert its action inside macrophages. To address this, we developed novel cationic nanostructured lipid carriers of efavirenz (cationic EFV-NLC).

Methods: The developed cationic EFV NLCs were evaluated for particle size, zeta potential, encapsulation efficiency, *in-vitro* drug release, DSC, XRD, TEM, cytotoxicity, cellular uptake studies and anti-HIV efficacy in a monocyte-derived macrophage cell line (THP-1).

Results: Cationic EFV-NLCs showed high encapsulation efficiency (90.54 ± 1.7%), uniform particle size distribution (PDI 0.3-0.5 range) and high colloidal stability with positive zeta potential (+23.86 ± 0.49 mV). DSC and XRD studies confirmed the encapsulation of EFV within NLCs. Cytotoxicity studies (MTT assay) revealed excellent cytocompatibility (CC_{50} 13.23 ± 0.54 µg/mL). Fluorescence microscopy confirmed the efficient uptake of cationic EFV-NLCs, while flow cytometry revealed time and concentration dependant uptake within THP-1 cells. Cationic EFV-NLCs showed higher retention and sustained release with 2.32-fold higher percent inhibition of HIV-1 in infected macrophages as compared to EFV solution at equimolar concentrations. Interestingly, they demonstrated 1.23-fold superior anti-HIV efficacy over EFV-loaded NLCs at equimolar concentrations.

Conclusion: Cationic NLCs were capable of inhibiting the viral replication at higher limits consistently for 6 days suggesting successful prevention of HIV-1 replication in infected macrophages and thus can prove to be an attractive tool for promising anti-HIV therapy.

Introduction

HIV/AIDS is a universal pandemic and is a key reason for mortality among adults worldwide.[1] According to the "Global HIV-AIDS Statistics-fact sheet 2019" by UNAIDS, since the commencement of the AIDS pandemic 74.9 million people have got infected with HIV. As per the 2018 data, there are 37.9 million HIV-positive adults and 1.7 million cases of new HIV infections across the globe.[2] Currently, any preventive vaccine or cure is not available for HIV-AIDS. HAART (highly active anti-retroviral therapy) has been able to reduce HIV-AIDS associated morbidity and mortality, but HAART needs strict adherence to the treatment regimen. However, HAART is also associated with significant side effects, possible risk of emergence of viral resistance and is ineffective in the thorough extermination of the virus from the body.[3]

Monocytes/macrophages are of crucial importance in HIV infection. Apart from their phagocytic function, they function as a safe-haven for ongoing HIV-replication.[4] It is observed that these cells act as latent reservoirs for HIV throughout the body, including organs such as the central nervous system, lymph nodes, kidneys, liver and etc.[5] These organs act as "anatomical viral reservoirs" where virus capable of replication can accumulate and stay there with more stable kinetic properties. Although HAART makes it possible to achieve effective control of HIV, it fails to eliminate the virus present in viral reservoirs. Therefore, effective anti-HIV therapy must be capable of delivering drugs to macrophages and anatomical reservoir sites.[6]

A significant technological gap exists in currently available anti-retroviral therapy and ideal features required for docile anti-retroviral therapy.[7] There are many drawbacks related to current anti-retroviral drugs, such as high dose,

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poor water solubility, low bioavailability, extreme first-pass metabolism, toxicity, low permeability, etc. Most of the current anti-retrovirals are unable to eliminate the virus from cellular and anatomical viral reservoirs owing to their poor availability at the reservoir sites, which eventually adds to its limited clinical outcome. Representation of efflux transporters (e.g., p-glycoprotein), poor permeability, non-targeted distribution, rapid clearance and metabolism by enzymes further complicates this issue.[8] Hence, once anti-HIV therapy is discontinued, or upon the development of drug resistance, HIV can replicate again and can spread the infection.[9] This suggests that a newer anti-HIV therapy should be capable of delivering drugs to the viral reservoirs. Efavirenz (EFV) (NNRTI, non-nucleoside reverse transcriptase inhibitor), is often recommended as a first-choice drug in HAART.[10,11] It is highly lipophilic and is categorized as BCS class II drug (low aqueous solubility and high permeability). Its pKa value is 12.52 and Log P value varies between 3.89-4.46. EFV shows high first-pass metabolism and has low aqueous solubility (0.00855 mg/mL), leading to poor bioavailability.[12,13] Previously, researchers have primarily focussed on attempts to enhance the oral bioavailability of EFV by developing micelles,[14] cubosomes,[15] polymeric nanoparticles,[16] SNEDDS,[17] nanoemulsion,[18] etc. However, the clinical outcome of the EFV treatment and toxicities are directly associated with low and high plasma concentrations of EFV, respectively.[19] Mostly, anti-HIV drugs are administered orally and are available as tablets, capsules, etc. Due to their intrinsic problems, viz. poor pharmacokinetic and pharmacodynamic properties, large doses are needed to be administered to attain the minimum effective concentration, which ultimately accentuates the threat of side effects, the emergence of viral resistance and other therapeutic complications. This suggests that improving bioavailability, reduction in the dosage and exploring non-oral routes of administration. Recently, Odongo *et al.*[20] have highlighted that parenteral anti-retroviral formulations are needed for better clinical outcomes. Therefore, there is a need for a drug delivery system capable of surmounting these challenges to deliver the drug to viral reservoir sites. Nanotechnology-based parenteral therapeutics can prove to be an attractive solution for this.

Nanotechnology-based approaches have presented revolutionary solutions for disease treatment. FDA has approved several nanotechnology-based products while few are in clinical trials.[3] One of the key hurdles in the case of anti-HIV nanotherapeutics is the solubility profile of the existing anti-HIV drugs, which hampers their encapsulation into nano vesicular systems. Lipidic nano systems such as SLN and NLCs have demonstrated high entrapment efficiencies. They offer control over formulation factors such as the nature of lipid, lipid ratio, lipid and surfactant concentrations. These factors can be modified to accomplish the desired quality target profile.[21] Newer lipidic carriers known as Nanostructured lipid carriers (NLC) are regarded as the most promising delivery system. In the shortest timeframe, they have transformed from invention to market introduction.[22] Structurally, NLCs consist of liquid lipid assimilated into a solid lipid matrix. NLCs offer numerous benefits such as higher drug loading, inhibition of drug crystallization, stability, low toxicity, controlled release, biodegradability and greener method of production.[23] Cationic nanoparticles can interact with the negatively charged cells via ionic interactions and thus can improve the uptake by the cells. They improve cell-nanoparticle contact due to electrostatic attraction and thus vesicular internalization.[24]

Based on the above scientific rationale, we envisaged that cationic nanocarriers capable of improving the availability of drugs at viral reservoir sites such as macrophages might prove to be an attractive strategy to overcome the drawbacks associated with current anti-HIV therapy. Such nanocarriers may help in a reduction in required doses for minimizing toxic effects. Hence, the rationale of the present research was to design EFV-encapsulating cationic NLC's capable of delivering drug to the cellular reservoirs (macrophages) to improve the anti-HIV efficacy as well as eradication of the virus from reservoir sites.

Statistical analysis

All the data obtained was expresses as the mean ± standard deviation (SD). ANOVA test was used as a tool to analyse the obtained data wherever applicable, followed by Dunnett's multiple comparisons test using Graph Pad Prism statistical analysis software (Version 7.0). $P < 0.05$ was considered as statistically significant ($P < 0.05$*, $P < 0.01$**, $P < 0.001$***, $P < 0.0001$****).

Materials and Methods

Materials

Efavirenz (EFV) was kindly provided by Hetero Labs Ltd, Hyderabad (India). Monosteol™ (Propylene glycol monopalmitostearate) was obtained from Gattefosse (France). Capmul® PG 8 (Propylene glycol monocaprylate) was obtained as a gift sample from ABITEC Corporation (USA). Tween® 80 (Polyethylene glycol sorbitan monooleate) was purchased from Merck Millipore, India. Poloxamer 188 (Pluronic® F-68) was procured from BASF, Germany. Milli-Q® water was used for the necessary preparations. Cetyltrimethylammonium bromide (CTAB), Coumarin-6, PMA (Phorbol 12-myristate 13-acetate), Interleukin 2 (IL-2), DMSO and Thiazolyl tetrazolium bromide (MTT assay) were obtained from Sigma-Aldrich. ActinRed 555 and 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) were obtained from Molecular Probes (USA). Trehalose was obtained from Signet Chemicals, India. Fetal Bovine Serum (FBS), Roswell Park Memorial Institute (RPMI) 1640 medium, Dulbecco's modified eagle medium (DMEM), Penicillin-Streptomycin, Fungizone were procured from Gibco (Invitrogen Corporation, USA). All other chemicals and reagents used were of an analytical grade.

Preparation of EFV loaded cationic nanostructured lipid carriers (cationic EFV-NLC's)

Preliminary screening of the solubility of EFV in various NLC components (solid lipid, liquid lipid (oils) and surfactants/co-surfactants.[25] surplus of EFV was added to each of the excipients, and the amount of EFV solubilized was determined using HPLC. Efavirenz loaded NLC's were prepared by following a green approach (avoiding the use of organic solvents), i.e., by using the hot-melt emulsification-ultrasonication method.[26,27] Concisely, a mixture of Monosteol™ (solid lipid, melting point 48°C-50°C) and Capmul® PG 8 (liquid lipid) was heated (10-15°C over the melting point of solid lipid) in a water bath to form a uniform melt (Lipid phase). Efavirenz (30 mg) was added to this preheated lipid phase and vortexed briefly using a vortex mixer in order to ensure complete mixing and solubilization. Concurrently, a surfactant solution (1.75% w/v) (in Milli Q water) heated to the same temperature, containing Tween 80: Poloxamer 188 (at predetermined ratio), was prepared. The preheated surfactant solution was added to the drug-containing lipid under vortex in order to ensure complete mixing and subjected to ultrasonication using a probe sonicator (Dakshin, Mumbai) for 120 seconds with a periodic on-off cycle (5-sec On:4 sec Off). The obtained EFV-NLCs were cooled to the room temperature. For the preparation of cationic EFV-NLCs, CTAB was used as a cationic surfactant. CTAB was introduced to the aqueous phase (1% w/w of lipid phase) to prepare an aqueous surfactant solution. Blank NLC's (without efavirenz) were prepared by following the same protocol.

Fluorescent NLC's were prepared by encapsulating coumarin-6 (Sigma-Aldrich, India) into the NLCs to prepare Cou-6 NLCs and cationic Cou-6-NLCs. The final concentration of coumarin-6 in the fluorescent NLCs was 2.5 ppm. EFV-NLCs and cationic EFV-NLCs were lyophilized (FreeZone4.5, Labconco Corporation, USA) using 1% trehalose as a cryoprotectant, and were used for characterization studies such as DSC and XRD.

Assessment of particle size and zeta potential

Particle size, PDI and zeta potential of prepared NLCs were measured using dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, UK). The samples were suitably diluted with Milli Q water, and measurements were done at a scattering angle of 90° at 25°C. The stability of NLCs at room temperature and culture medium was evaluated by measuring particle size, PDI and zeta potential. All the measurements were performed in triplicate and reported as mean ± standard deviation (S.D.).

Drug loading and drug encapsulation efficiency

The amount of drug encapsulated in NLCs was determined in order to estimate the drug loading and drug encapsulation efficiency. Briefly, a measured quantity of NLC's formulation was centrifuged at 35,000 rpm (Thermo Sorwall WX Ultra, USA) at 20°C for 1 h. The supernatant and settled lipid pellets were carefully separated and dissolved in an appropriate quantity of methanol. Estimation of the amount of EFV present in the supernatant as well as in lipid pellet was determined using previously reported HPLC method with slight modifications.[28] Chromatography was performed using ODS C-18 Column (Phenomenex, Luna; 4.6 mm X 250 mm, 5µ). The mobile phase comprising of acetonitrile and ammonium phosphate buffer in the 60:40 v/v (pH 3.5) was pumped at a flow rate of 1.5 ml/min. The detection was performed at the wavelength of 245 nm using the HPLC system equipped with a PDA detector, Jasco PU2080 plus (Intelligent HPLC) Pump with and autosampler unit. Encapsulation efficiency and drug loading were calculated using the following equations:

$$\text{Encapsulation efficiency (EE\%)} = \left(\frac{Wa - Ws}{Wa}\right) \times 100 \qquad \textbf{Eq. (1)}$$

$$\text{Drug loading (L\%)} = \frac{We}{WL} \times 100 \qquad \textbf{Eq. (2)}$$

Where Wa= amount of drug added to the formulation,
Ws= amount of free drug
We= actual amount of drug encapsulated
WL=Weight of lipid

Differential Scanning Calorimetry

The physical state and polymorphism in the prepared NLCs were studied with the help of Differential Scanning Calorimetry (DSC) using Pyris 6 DSC (Perkin Elmer, USA). Weighed amount (approx. 3-5 mg) of samples were put in standard aluminium and sealed. The samples were scanned at a constant heating and cooling rate of 5°C/min under nitrogen gas for the temperature from 30° C to 250 °C. Sealed empty aluminium pan was kept as a reference.

X-ray diffraction analysis

Powder X-ray diffraction patterns of EFV, physical mixture (a mixture of all NLC components prepared by simple mixing) and lyophilized NLCs (EFV-NLCs and cationic EFV-NLCs) were recorded using Philips X'Pert MPD Diffractometer (Philips, Netherlands) run at voltage of 40 kV and a current of 20 mA. The scanning speed was fixed at 2° per min while exposing the samples to Cu-Kα radiation over a range of 2θ angles from 2° to 60°.

Transmission electron microscopy

Morphologic evaluation of NLC's was performed using Transmission Electron Microscopy (TEM) (Tecnai-20, Philips, Holland). NLCs were diluted suitably using Milli Q water before the analysis and a small drop of NLC was placed on carbon-coated copper grids followed by the addition of a drop of 2% (w/v) uranyl acetate. Images were captured by mounting the grid on the instrument after draining off excess liquid and air-drying.

In vitro release study

The amount of drug released from the NLCs over the period was estimated by performing *in-vitro* release studies using a dialysis bag method. Pure drug suspension (5 mg of EFV suspended in PBS) and EFV-NLCs, cationic EFV-NLCs equivalent to 5 mg of EFV were introduced in the dialysis bag membrane (MW cut off 12-14kDA, Himedia). The sealed dialysis bag was submersed in the dissolution medium (Phosphate buffer saline pH 7.4, 200 mL) kept at stirring speed of 50 rpm for 24 h at 37° C. Sink conditions were maintained throughout the release studies. At predetermined intervals, the sample was withdrawn from the vessel and diluted suitably for estimation of the amount of EFV by HPLC, as previously described. *In-vitro* drug release from NLC's and pure drug suspension was assessed against each other.

Cell culture

Human Monocytic THP-1 cell line (THP-1 cells) and HEK 293T cells were obtained from the National Institutes of Health (NIH), USA through NIH AIDS Reagent Program, Division of AIDS, NIAID.

The adherent HEK 293T cell line was used for the production of infectious virus particles. The cells were kept in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) supplemented with 10% FBS, 25 mM HEPES solution (Gibco, Invitrogen, USA) and 100 units/mL each of penicillin G and streptomycin (Gibco, Invitrogen, USA). The cells were cultured in an incubator at 37 °C in a humidified 5% CO_2 atmosphere and were used at 80% confluency.

Monocytic THP-1 cell line was maintained in RPMI 1640 (Gibco, Invitrogen, USA) complete growth medium, supplemented with 10% FBS, 25 mM HEPES solution (Gibco, Invitrogen, USA) and 100 units/mL each of penicillin G and streptomycin (Gibco, Invitrogen, USA), in an incubator at 37 °C in a humidified 5% CO_2 atmosphere and was used after attainment of 80% confluency. Monocytic THP-1 cells were differentiated into macrophages by resuspension in THP-1 medium, supplemented with 100 ng/mL PMA (Phorbol 12-myristate 13-acetate) seeded at a density of 1 X 10^6 cells per well or per coverslip in Corning™Costar™ tissue culture plates for 24 h, in an incubator at 37 °C in a humidified 5% CO_2 atmosphere. Following this, THP-1 media was removed and washed with RPMI 1640 medium and let 24 h at 37°C in fresh complete RPMI 1640 medium before experiments. Monocytic THP-1 cells adhere to the surface of coverslips and wells upon differentiating into THP-1 macrophages.

Virus culture

Infectious molecular clone for NL4.3 virus was procured from NIH, USA, through NIH AIDS Reagent Program, Division of AIDS, NIAID. The plasmid was maintained in TOP10 (*E. coli* DH5 alpha, Invitrogen, USA). Before culturing the virus, *E. coli* was grown in Luria Bertani broth supplemented with 100 µg/mL of ampicillin overnight at 37°C. The plasmid was extracted using QIAprep™ spin miniprep kit (Qiagen, USA) and the concentration of the plasmid was assessed on the Nanodrop UV spectrophotometer. For the production of infectious virus, 1.5 µg of plasmid DNA was transfected into HEK293T (4x 10^5cells/well) in a 6 well tissue culture plate (Thermo Fischer Scientific, USA) using a Calcium phosphate transfection kit (Invitrogen, USA) by following the manufacturer's protocol. The virus released in the culture supernatant was harvested, centrifuged, filtered through a 0.22 µm syringe filter and stored at -80°C until further use. The developed stock was titrated as reported previously[29] in differentiated THP-1 cells to calculate the multiplicity of infection (MOI) for anti-HIV assays.

Cytotoxicity studies by MTT assay

Cell viability of THP-1 cells upon treatment with EFV, blank NLC's, EFV-NLC's and cationic EFV-NLCs was measured by using MTT assay. 100 µL of cell suspension was seeded into each well of a 96-MicroWell™ plate (0.2 X 10^5/well) (Thermo Fisher Scientific, USA). Monocytic THP-1 cells were allowed to differentiate into macrophages by suspending them into THP-1 media, as described above. The samples of EFV, Blank NLC's EFV-NLC's and cationic EFV-NLCs (concentrations equivalent to 150 µg/mL to 2.34 µg/mL of EFV corresponding to 45µM to 0.70µM , respectively) were introduced to the wells and the plate was kept in an incubator at 37°C in 5% CO_2. THP-1 cells were incubated with respective samples for 6 days. Following this, the media from each well replaced with MTT solution (5 mg/mL MTT in PBS) and incubated for 4 h at 37°C in 5% CO_2. Later, the MTT solution was carefully removed from each well completely without disturbing the formed formazan crystals. The formazan crystals were dissolved by adding 50µl of DMSO to each well and plate was incubated at room temperature under dark, for 30 mins. The absorbance was measured at 550 nm and 690 nm in a PowerWave microplate spectrophotometer (Biotek Instruments Inc, USA). The results were calculated by non–linear regression curve fitting and expressed as CC_{50} (concentration at which 50% cells are viable).

Fluorescence microscopy

Fluorescence microscopy was used to understand and study the uptake of fluorescent NLCs by the cells. Coumarin-6 (fluorescent dye) was used as a marker and was encapsulated into NLCs to investigate the cellular uptake of NLCs. Fluorescent NLCs were subjected to dialysis before performing fluorescence microscopy to remove the unencapsulated coumarin-6 dye. Briefly, 6-well culture plates were used and each well was seeded with cells in order to get 1 X 10^6 cells per well on sterile glass coverslips. Monocytic THP-1 cells were differentiated into macrophages by suspending them into THP-1 media as described above, to facilitate adhering to coverslips. THP-1 media form each well was then removed and was replaced with 1 ml of coumarin-6 loaded NLC's diluted

in RPMI 1640 medium. The subtoxic concentration of fluorescent NLC's along with cells was incubated at 37°C and 5% CO_2 for a period of 4 h. After incubation, complete removal of media was done and the cells were treated with 4% paraformaldehyde solution for 15 mins. The cells were washed 3 times with PBS and 0.1% Triton X solution was added and incubated for 3 mins. Later, Triton X solution was removed and cells were washed thrice with PBS. Then the cells were stained with ActinRed 555 ReadyProbes (cellular membrane stain, excitation/emission (nm):540/565) and DAPI (4′,6-Diamidino-2-phenylindole dihydrochloride) solution (nuclear stain; excitation/emission (nm): 358/461 nm) at the same time. During this procedure, the cells were maintained in an incubator for 30 mins at 37°C and 5% CO_2. Following this, the cells were washed thrice with PBS, mounted on glass slide and observed under Fluorescent microscope (EVOS M5000, Invitrogen, USA). All the experimental steps were performed under low light conditions to avoid fluorescent dequenching.

Flow cytometry

Uptake of NLC's by the THP-1 cells was further studied by using FACS analysis (Flow cell Cytometry, BD FACS Aria, BD Biosciences, USA). Monocytic THP-1 cells were differentiated into macrophages as stated previously. The cell suspension was seeded to each well of 6-well plate containing coverslips, at a cell density of 1 X 10^6 cells per coverslip in Corning™Costar™ tissue culture plates for 24 h, in an incubator at 37 °C and 5% CO_2 atmosphere. These cells were treated with coumarin-6 loaded NLC's at 1 µM and 10 µM concentration, for 2 h, 4 h, 6 h, and 8 h at 37 °C and 5% CO_2 atmosphere. After the incubation, the medium was removed and the cells were washed thrice with PBS. The cells were detached from coverslips by scrapping, resuspended in PBS and subsequently analyzed with FACS Aria (BD Biosciences, USA) at 443 nm. Flow cytometry data was analyzed using FACSDiva™ Software.

Anti-HIV efficacy by p-24 antigen assay

To study and observe the *in vitro* anti-HIV efficacy of cationic EFV-NLC's p24-antigen assay was performed. Monocytic THP-1cells were differentiated into macrophages by suspending them in the THP-1 medium, as described previously. HIV-infection was carried out by infecting THP-1 cells with HIV-1 NL4-3 titrated virus stocks (0.01MOI). Cells were incubated for 6 h at 37°C and 5% CO_2. Following this, the medium was removed from each well and the cells were washed at least thrice with fresh RPMI 1640 media. Cells were then treated with respective anti-HIV formulations (EFV drug and EFV-loaded NLC's) at concentrations of 1 µM, 10 µM and 25 µM. The supernatant from individual wells were collected at day 2, day 4 and day 6. The supernatants collected were analyzed for the detection of p24 antigen in order to determine the anti-retroviral efficacy of prepared nanocarriers. ELISA was performed using the HIV-1 p24 Antigen Capture Assay kit (ABL Inc, USA). Mock infected cells and untreated HIV-1 infected cells were maintained as controls in each experiment.

Results and Discussion

Preparation of EFV loaded nanostructured lipid carriers

NLC's are popularly known as second-generation solid lipid nanoparticles or SLN's. In lipidic carriers such as NLCs, selection of each excipient for achieving maximum stability, optimum particle size and maximum drug loading is critical. Screening of the several excipients was therefore carried out carefully in order to finalize each excipient.

Efavirenz belongs to BCS class II and is a poorly water-soluble drug.[12] During preliminary screening studies, among the several solid lipids and liquid lipids screened; EFV showed maximum solubility in Monosteol (Propylene glycol monostearate) and Capmul PG 8 (Propylene glycol monocaprylate), respectively (see supplementary information). Monosteol and Capmul PG 8 were chosen as a solid lipid and liquid lipid (oil) for NLC formulation. The chemical nature of lipids plays an important role in the stability of the colloidal systems as well as drug expulsion. Lipids selected for NLC preparation should have maximum solubility for the selected drug. Lipids, which are mixtures of mono-, di- and triglycerides and lipids which have a mixture of fatty acids that have different chain lengths offer more stability and higher drug encapsulation.[30] Monosteol and Capmul, as a heterogeneous mixture of fatty acids, resulted in better solubilization of EFV within them. Expulsion of the drug from the colloidal system is a major drawback of lipidic colloidal systems. It is advantageous to have solid lipid and liquid lipid components such that, the drug remains solubilized to the maximum extent in these two components during formulation and storage. This helps in minimizing the drug expulsion along with better encapsulation and loading efficiency.[31,32] Monosteol and Capmul showed good compatibility and no drug expulsion was noticed upon EFV solubilization in them. Besides this, over the storage period there was no phase separation.

Non-ionic surfactants are less toxic, biocompatible and have good parenteral acceptability.[33] Surfactants were screened in the concentration range from 1% to 2%. Surfactant ratio and concentration which helped in achieving low PDI (0.3-0.4 range), no phase separation along with no formation of viscous solution over the period of time were selected for NLC preparation. Among various surfactants screened, poloxamer 188 and tween 80 showed good potential to solubilize EFV. Both these excipients are reported to enhance the stability of the system by steric stabilization and increase electrostatic repulsion potential energy among the particles.[34] The high HLB value of poloxamer 188 also helps in achieving a colloidal system with lower particle size.[35] Surfactant concentration plays an important role in the stabilization of the colloidal systems. Higher amounts of surfactant significantly affect particle size and result in lower PDI.[36] At the same time, higher surfactant concentrations are harmful to cells and can result in

increased cytotoxicity. Various ratios of solid lipid and oil, surfactant concentrations were investigated to evaluate the physical compatibility, stability and drug expulsion. Based on the primary formulation batches, we observed that total lipid concentration, surfactant concentration and drug concentration are the major factors which affect the particle size, stability and encapsulation efficiency of the NLCs. Hence, we decided to evaluate the effect of these factors on particle size, stability and encapsulation efficiency of the NLCs. We observed that, linear relationship exists between particle size and total lipid concentration and particle size increases upon increasing the total lipid concentration. Also, we observed that, as an individual surfactant (and not present in combination with each other) poloxamer 188 or tween 80 were not able to impart stability to the system and achieve optimum particle size. The particle size increased even after increasing the surfactant concentration to the highest levels. The major factors which shown a significant effect on entrapment efficiency were total surfactant concentration including poloxamer 188: tween 80 ratio. As the surfactant concentration decreased, the entrapment efficiency decreased accompanied by increase in the particle size and instability. Based on the preliminary screening, observations and obtained results, the percentage of Monosteol: Capmul PG 8 was fixed to 71.5% w/w and 28.5% w/w of the total lipid load. Total surfactant concentration was fixed to 1.75% w/v, with Poloxamer 188 and Tween 80 concentrations 1.3% w/v and 0.43 % w/v. For the preparation of cationic NLCs, CTAB was used as a cationic surfactant. CTAB concentration was fixed at 1% w/w of the total lipid phase.

The optimum concentration of surfactants and selection of lipid ratio is critical for stabilization of the NLC system as well as in achieving optimum encapsulation efficiency of the drug. Previous reports suggest that, increasing surfactant concentration up to certain levels can result in increased encapsulation efficiency. However, beyond certain levels, it does not affect the amount of drug entrapped and may lead to some undesired results.[37,38] We, therefore, selected surfactant ratio, concentrations, lipids and lipid ratio on account of overall stability, particle size and distribution and encapsulation efficiency of the system. Surface charge and surface modification plays an important role in the overall functionality of a nanomedicine. Incorporation of cationic surfactants is done for improving the cell uptake, opening of tight junctions, improved gene conjugation and increased drug loading. Cationic nanomedicines prove to be useful in cell targeting, as they have the advantage of superior interactions and uptake by negatively charged cells due to electrostatic interactions. Thus, we modified the surface charge of the prepared NLCs with CTAB to help the interaction between NLCs and cell membrane. Hot emulsification homogenization method was selected for the preparation of NLCs due to the intrinsic advantages it offers. NLCs and SLN can be prepared using methods such as high-pressure homogenization, emulsification/solvent evaporation, emulsification/solvent diffusion, high-speed homogenization, supercritical fluid extraction, etc. In high-pressure homogenization, two techniques are available, i.e. hot homogenization and cold homogenization. We adopted hot emulsification homogenization method (Hot homogenization) for the preparation of NLC's as it is simple, avoids the use of organic solvents and scalable. It is well established and potential technique that can be used for large-scale production, unlike the other available techniques.[39]

Particle size measurement and zeta potential

Particle size and zeta potential play a critical role in the stability of colloidal solutions. Lesser particle size and less PDI are always desired as quality attributes and considered as the hallmarks of a stable colloidal system. The particle size and zeta potential of the prepared NLCs (EFV NLCs and cationic EFV-NLCs) is shown in Figure 1. Colloidal

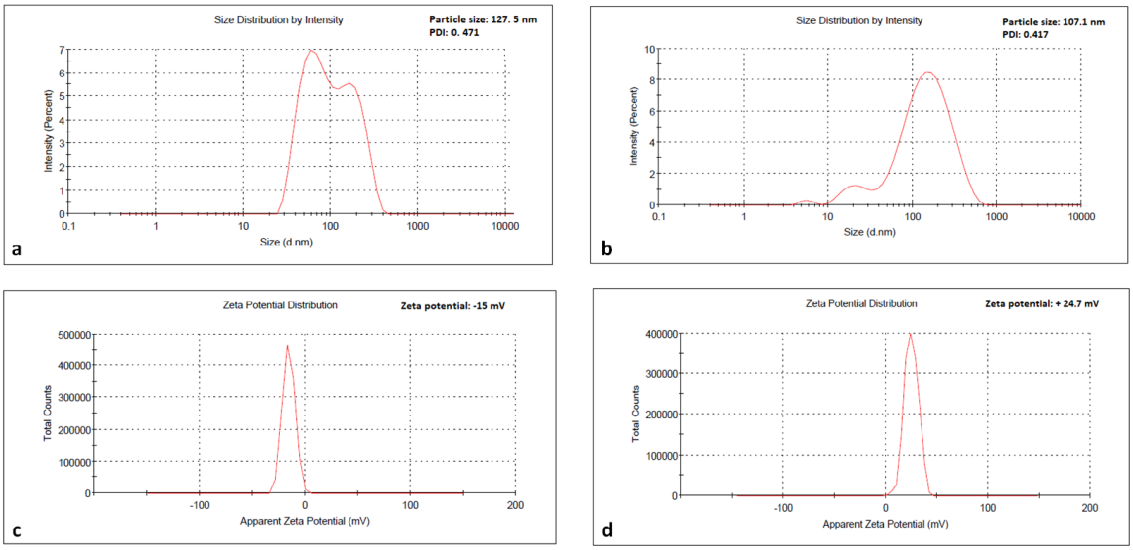

Figure 1. Particle size and zeta potential of NLCs, a. particle size of EFV-NLCs, b. particle size of cationic EFV-NLCs, c. zeta potential of EFV-NLCs and d. zeta potential of cationic EFV-NLCs.

Table 1. Particle size analysis of EFV-NLCs and cationic EFV-NLCs.

No.	Sample Name	Particle size (nm)	Zeta potential (mV)	PDI
1	Blank NLCs (in Milli Q water)	114. 53 ± 5.63	-15.16 ± 0.69	0.449 ± 0.02
2	EFV NLC (in Milli Q water)	116.5 ± 9.59	-15.8 ± 1.21	0.434 ± 0.13
3	EFV NLC (in culture medium) EFV NLC (after 10 days in culture medium)	124.1 ± 8.82 126.16 ± 5.98	-16.1 ± 0.67 -15.8 ± 1.02	0.44 ± 0.06 0.412 ± 0.03
4	Cationic EFV NLC (in Milli Q water)	105.6 ± 4.93	+23.86 ±0.49	0.417 ± 0.03
5	Cationic EFV NLC (in culture medium) Cationic EFV NLC (after 10 days in culture medium)	106.5 ± 2.76 105.53 ± 2.44	+23.83 ± 0.20 +24.3 ± 0.65	0.407 ± 0.01 0.417 ± 0.01
6	Coumarin-6 NLC (in Milli Q water)	124.8 ± 7.93	-16.2 ± 1.44	0.464 ± 0.02
7	Coumarin-6 NLC (in culture medium) Coumarin-6 NLC (after 10 days in culture medium)	130.9 ± 2.59 127.23 ± 7.74	-15.9 ± 0.92 -15.6 ± 1.51	0.452 ± 0.03 0.405 ± 0.02
8	Cationic Coumarin-6 NLC (in Milli Q water)	105.23 ± 2.73	+24.2 ± 1.01	0.416 ± 0.01
9	Coumarin-6 NLC (in culture medium) Coumarin-6 NLC (after 10 days in culture medium)	108.33 ± 2.44 109.56 ± 2.94	+24.2 ± 0.787 +23.7 ± 1.04	0.452 ± 0.03 0.403 ± 0.02

systems are regarded as more stable if zeta potential values are sufficiently high, according to DLVO theory.[40] Table 1 summarizes the average particle size, zeta potential and PDI of the prepared NLCs. Particle size of the blank NLCs, EFV-NLCs and cationic EFV-NLCs was found to be 114. 53 ± 5.63 nm, 116.5 ± 9.59 nm and 105.6 ± 4.93 nm, respectively. We used a combination of non-ionic surfactants; poloxamer 188 and tween 80, along with CTAB (to impart positive charge) to stabilize colloidal NLCs. The zeta potential of blank NLCs and EFV-NLCs was found to be -15.16 ± 0.69 mV and -15.8 ± 1.21 mV, respectively while zeta potential of cationic EFV-NLCs was found to be +23.86±0.49 due to incorporation of CTAB. Non-ionic surfactants cannot ionize like ionic surfactants but exhibit a zeta potential and are able to stabilize the system attributable to molecular polarization, steric repulsion and adsorption of emulsifier molecule on the particle/water interface. Additionally, poloxamer 188 has typical surfactant properties and is able to assemble around the surface of nanocarrier and form a complex micelle due to poly(propylene oxide) and poly(ethylene oxide) chains.[34] Cationic EFV-NLCs showed lower particle size than EFV-NLC due to the incorporation of CTAB. The loading of cationic surfactants has a significant effect on particle size. CTAB exerts an emulsifying effect upon incorporation into lipid-based nanosystems which results in a decrease in the particle size.[41] It is essential to ensure the stability of prepared NLCs in the culture medium. For this purpose, we measured the particle size of the prepared NLC in the presence of culture media in order to investigate if the prepared NLC's agglomerate or aggregate during *in-vitro* experiments. All the prepared NLCs showed uniform particle size distribution. Poloxamer 188 and tween 80 stabilized NLCs showed negative zeta potential; while upon the incorporation of CTAB, NLCs with positive zeta potential were obtained. The zeta potential of prepared NLCs (EFV-NLCs and cationic EFV-NLCs) was sufficiently high, indicating the colloidal stability. Hence from the obtained results, it can be inferred that there is no aggregation and significant variations in the particle size, zeta potential and PDI upon dilution in a culture medium, indicating the stability of prepared NLCs.

Drug loading efficiency and drug encapsulation efficiency

Drug loading efficiency and encapsulation efficiency are important factors in NLC development. Encapsulation efficiency and drug loading efficiency were computed by using equation 1 and 2, respectively, as stated previously. The encapsulation efficiency of prepared EFV NLC's was found to be 91.18 ± 2.9 % with drug loading efficiency 10.94 ± 0.35 %. Upon incorporation of CTAB as a cationic surfactant, encapsulation efficiency did not change significantly. Cationic EFV-NLCs showed 90.21 ± 2.3% with drug loading efficiency 11.04 ± 0.17%. The decrease in the particle size was noted upon CTAB incorporation; however, this did not hamper the encapsulation efficiency and drug loading efficiency. The primary requirement for achieving high encapsulation efficiency and optimum loading efficiency in NLCs is the solubility of the drug in lipids.[42] High encapsulation efficiency obtained for the prepared NLCs can be assigned to superior solubility of EFV in lipids. EFV is a class II molecule and has high lipophilicity, which results in more solubility in lipids. Monosteol and Capmul PG 8 belong to the class of modified mixed chain fatty acids. Mixed chain fatty acid derivatives of glycerides provide more accommodation for lipophilic drugs.[30] Higher amount of drug encapsulated in the NLC system provides drug release in a sustained manner, while small fraction present as a free drug in surfactant micelles provides quick bioavailability.[25]

Differential scanning calorimetry

DSC studies of the pure drug (EFV), drug lipid physical mixture, EFV-NLCs and cationic EFV-NLC were also performed (Figure 2). DSC thermogram of the pure drug (EFV) showed a sharp peak at 138.46 °C and the drug-lipid physical mixture showed a small peak around 145.6 °C, which indicates the melting point of the pure drug. Sharp melting endotherm for the EFV in both the DSC thermograms indicates the presence of the drug in crystalline form. In the DSC thermogram of the drug-lipid physical mixture, the endothermic peak was observed at

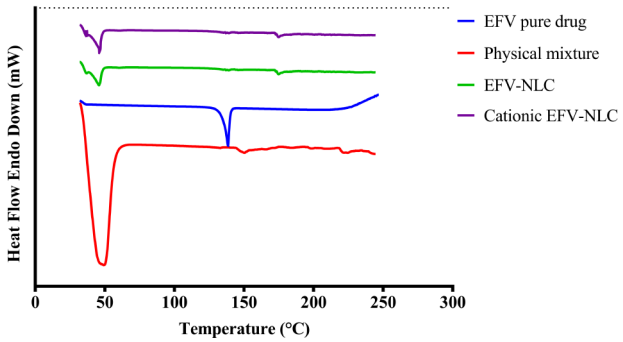

Figure 2. DSC thermograms for EFV pure drug, physical mixture, EFV-NLC and cationic EFV-NLC formulation.

48.8 °C, corresponding to the melting point of Monosteol. We observed that the DSC thermograms of EFV-NLC and cationic EFV NLC were almost similar and are devoid of any sharp peak corresponding to the melting endotherm of the drug, indicating that EFV is molecularly dispersed in the lipid matrix.[43] These thermograms appeared superimposed and indicated endothermic peak for Monosteol at 42.6 °C (shift from 48.8 °C). This reduction in the melting enthalpy can be ascribed to the nanosize of the colloidal particles as well as the formation of lower ordered lattice structure during lipid recrystallization in colloidal systems.[44,45]

X-ray diffraction analysis

With the purpose of studying the physical state of EFV encapsulated in NLC, XRD analysis of pure drug, drug lipid physical mixture, EFV-NLC and cationic EFV-NLC was performed. X-ray diffraction pattern (Figure 3) of the pure drug (EFV) exhibited typical peaks at 2θ values ranging from 7.1 to 32.24, with sharp peaks at 2θ of 17.66, 20.9, 21.2, 24.9 and 32.44 indicating its crystalline nature. XRD pattern for drug-lipid physical mixture showed peaks at 2θ values ranging from 13.8 to 24.9, corresponding to EFV drug with several other peaks corresponding to other formulation components. The XRD-diffraction pattern for both the prepared NLCs didn't show any sharp peaks corresponding to the typical crystalline nature of EFV, indicating that EFV is molecularly dispersed and incorporated with NLC structure.

Transmission electron microscopy

Surface morphology studies of EFV-NLC (Figure 4a)

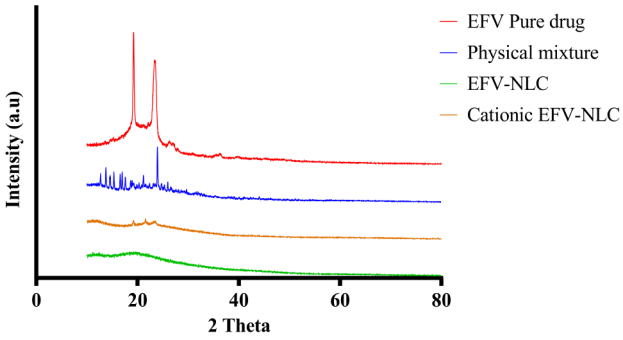

Figure 3. XRD patterns for EFV, Physical mixture, EFV-NLC and cationic EFV-NLC formulation.

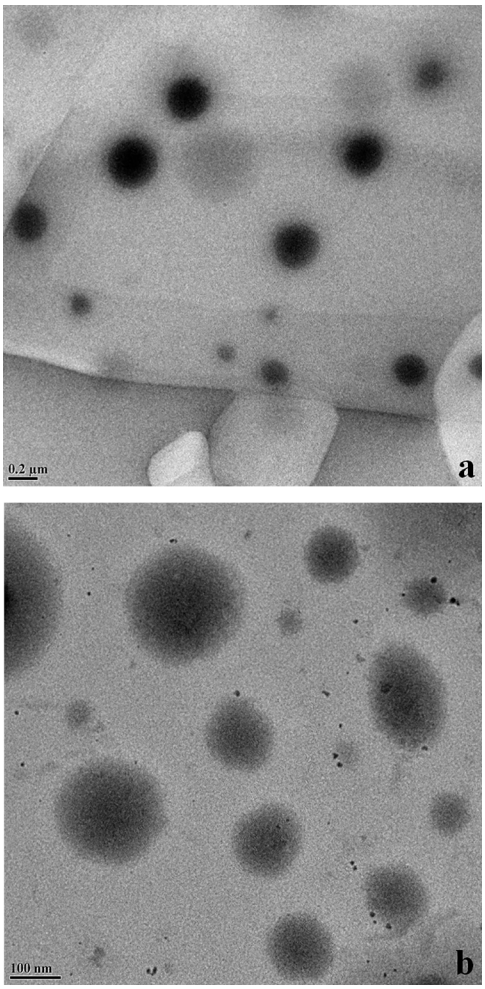

Figure 4. a) Transmission electron micrographs of EFV-NLC, and b) cationic EFV-NLC (b).

and cationic EFV-NLC (Figure 4b) were done with the help of Transmission electron microscopy. TEM revealed spherical shaped NLCs with a smooth surface. Both the NLCs demonstrated similar morphology with no distinct differences. Results obtained were in agreement with the results of particle size measurements which revealed a nanometre size of NLCs with uniform particle size distribution. However, TEM analysis may reveal particle size different than that determined by DLS method. The possible explanation for this phenomenon is, the actual size of particles which are in non-hydrates state is measured in TEM analysis as opposed to DLS method, where the hydrodynamic radius of the particle present in a hydrated state is measured.[46]

In vitro release study

In-vitro release studies of pure drug, EFV-NLC and cationic EFV-NLC were carried out in PBS pH 7.4 buffer. It was observed that upon encapsulation into NLC, the solubility of EFV is improved and the drug is released in a sustained manner over a period of time. For pure drug (EFV suspension in PBS, pH 7.4) cumulative drug released was 42.23 ± 1.84 %, whereas drug released from

EFV-NLC's and cationic EFV-NLCs, it was 69.76 ± 3.34 % and 67.36 ± 3.56 % respectively, after 24 h in PBS pH 7.4 as shown (Figure 5). EFV belongs to BCS class II and has poor solubility and thus the amount of drug released from EFV-suspension remained low throughout the study period attributable to its low wettability in suspension form. In EFV suspension we didn't use any solubilizing aid such as surfactant, solubilizing solvent etc. Therefore, in the absence of any solubilizing aid, the cumulative release from EFV suspension is purely dependent upon intrinsic solubility of EFV in the suspension medium.

At the end of 1 h, cumulative drug release from pure drug (EFV suspension in PBS, pH 7.4) was 8.63 ± 0.78 % whereas for EFV-NLCs and cationic EFV-NLCs it was found to be 17.93 ± 2.73% and 20.1 ± 3.74%, respectively. NLC formulations showed a typical biphasic pattern of drug release with initial burst release. The drug released from EFV-NLCs and cationic EFV-NLCs followed a similar pattern. Except for zeta potential and particle size, cationic EFV-NLCs did not differ in terms of composition from EFV-NLCs. Both the NLCs showed high encapsulation efficiency for EFV; however, some amount of unencapsulated drug is always present in nanoformulations. This free drug is present in an adsorbed form on the surface of the nanoparticle or is present in surfactant micelles or it may get precipitated from superficial lipidic matrix of NLC structure. This amount of free drug contributes to the initial burst release from the nanoformulations.[13,25] EFV-NLCs exhibited sustained release over the period of time after the initial burst release. EFV is a highly lipophilic drug and thus it remains well encapsulated within the lipid matrix; also the presence of hydrophilic surfactant in NLC systems modulates the drug release from the lipid matrix which mainly takes place by diffusion mechanism.[47,48] This contributes to the prolonged release of EFV from NLC's. The release rate of EFV from EFV-NLCs and cationic EFV-NLCs is significantly enhanced as compared to pure drug. Nanoformulations have low particle size, which contributes to enhanced surface area and helps in improvisation of drug wettability as well as the reduction of diffusion layer thickness.[49] This contributes to the improvisation of solubility, together with the release rate of the drug from nanoformulations.

Cytotoxicity studies by MTT assay

In order to investigate if the developed nanoformulations are toxic towards THP-1 cells, comparative cytotoxicity studies (n=3) of with pure drug (EFV), blank NLCs, EFV-NLCs and cationic EFV-NLCs were performed by MTT assay (Figure 6). The results were presented as CC_{50} value (concentration at which 50% cells are viable) and is computed by regression analysis. CC_{50} values for EFV, blank NLC, EFV NLC and cationic EFV-NLC were found to be 17.12 ± 0.44 µg/mL, 14.50 ± 0.29 µg/mL, 14.38 ± 0.47 µg/mL and 13.24 ± 0.54 µg/mL, respectively ($P < 0.05$). From the results, it can be observed that, cytotoxicity values of NLCs are significantly higher as compared to EFV alone, even in the case of blank NLCs. Nanoparticles are able to reduce the cytotoxicity of the drug, but opposite phenomenon was observed in our studies. In terms of cell viability, at the highest concentration of 150 µg/mL, every formulation proved to be lethal to the cells and cell viability values were too low. At 18. 75 µg/mL concentration, the cytocompatibility of pure drug (EFV) appeared to improve significantly ($P<0.05$) as compared to NLCs (blank NLCs, EFV-NLCs and cationic NLCs) where they all proved to be cytotoxic to the cells (Figure 6).

Cytotoxicity studies focus on the activity of drug[50,51] however blank nanoparticles are also able to exert cytotoxicity on cell lines. Cytotoxicity induced by NLC's is dependent upon lipid-core components as well as a surfactant.[52] Monosteol (Propylene glycol monostearate), is chemically a derivative of glyceryl monostearate. Derivatives of glyceryl monostearate cause an imbalance in the oxidative metabolism of the cells by causing changes in intracellular levels of ROS and thus can contribute to mild to moderate cytotoxic effects on cells.[53] The cytotoxicity observed in case of blank nanoparticles can be assigned to the rise in levels of ROS due to the presence of monosteol. When compared to blank NLCs cytotoxicity exerted by EFV-NLCs and cationic EFV-NLCs appeared similar and non-significant ($P < 0.05$). This indicated that no additional changes occur in the safety profile of NLCs upon encapsulation of EFV into NLCs and Monosteol is the main component which contributes to the cytotoxic effect associated with the NLCs. Interactions between nanoparticles and the cell membrane are affected by

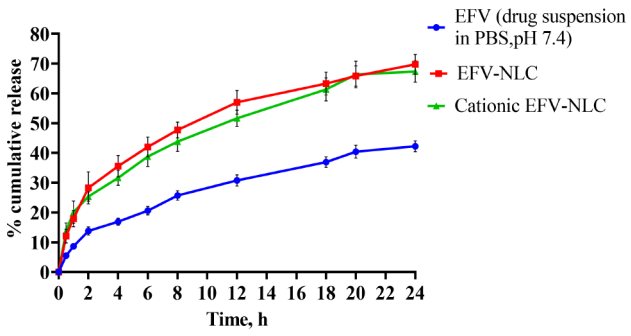

Figure 5. *In-vitro* drug release studies of EFV drug suspension, EFV-NLCs and cationic EFV-NLCs in PBS pH 7.4.

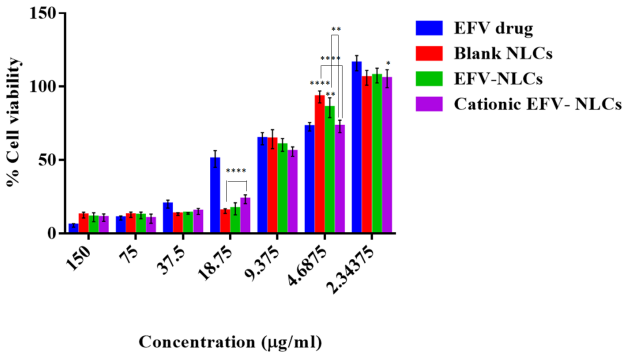

Figure 6. Cytotoxicity of EFV, EFV-NLCs and cationic EFV-NLCs against THP-1 macrophage cell line.

the molecular structure of surfactants at the interface of nanoparticles. CTAB, which was used to impart positive charge to the NLCs can exert cytotoxic action either on cells via activation of polymorphonuclear neutrophils (PMNs) which can secrete cytokines and chemokines responsible for initiation of the inflammatory response. This might result in cell death owing to the production of oxidative stress and stimulation of inflammation.[41] However, cationic NLCs exerted no additional cytotoxicity even upon incorporation of CTAB into them. With the help of the cytotoxicity studies, the safety profile of the prepared NLCs was established and results indicate that prepared NLCs does not induce major harmful effects on the cells.

Fluorescence microscopy

The interactions between prepared NLCs and cell membrane, cellular uptake and ultimately, their ability to cross the cytoplasmic membrane was investigated with the help of fluorescence microscopy (Figure 7). Fluorescent NLCs was subjected to dialysis in order to ensure that there is no excess of fluorescent dye, which will give misleading results. Cells were incubated with free coumarin-6 (control), coumarin-6 loaded NLCs and cationic coumarin-6 loaded NLCs separately using the same protocol. Cells treated with free coumarin-6 solution did not give any green fluorescence as cells do not uptake free coumarin-6 and is eliminated during the processing of cells.[54] This confirmed that any component of lipid matrix doesn't have native fluorescence and free coumarin, if present in NLCs, has not interfered with the fluorescence microscopic studies. On the other hand, cells treated with Coumarin-6 loaded NLCs showed intense intracellular green fluorescence. DAPI stained nucleus which can be seen in blue, while ActinRed 555 stained cytoplasm which can be seen in red colour. It can be observed that after incubation of cells with coumarin-6 loaded NLCs, all of the cells showed green fluorescence confirming the nanoparticles uptake by cells. Uptake of nanoparticles by cells occurs either by endocytosis via clathrin- and caveolin-mediated routes, macro-pinocytosis, pinocytosis or phagocytosis. In our experiments, green fluorescence was observed in most of the areas within the cells, highlighting efficient uptake potential of prepared NLCs by the cells. This also suggests that the release of the model drug (coumarin-6) occurs after the uptake of nanoparticles by the cells. For a drug delivery system, it is always desirable that the lipophilic drug (in our study, efavirenz) gets transported to the targeted area and is released at the desired site for effective pharmacological activity. For quantitating the uptake of the lipophilic drug, flow cytometry was performed.

Flow cytometry

In order to determine the quantitative uptake of nanoparticles by monocyte-derived macrophages, flow cytometry was performed. The fluorescence intensity of NLCs uptake by the cells was determined. Coumarin-6 loaded NLCs (mimicking EFV-NLCs) (Figure 8a) and cationic coumarin-6 loaded NLCs (mimicking cationic EFV-NLCs) (Figure 8b) were incubated with the cells with the purpose of understanding the characteristics and

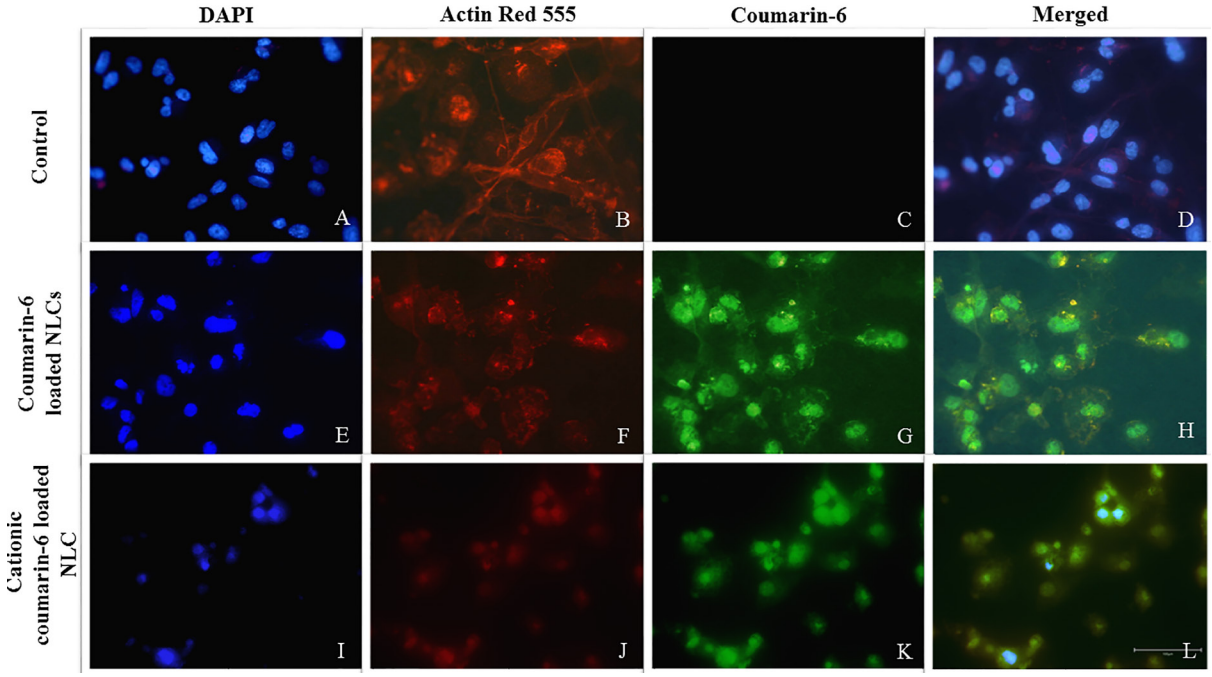

Figure 7. Fluorescence microscopy images for coumarin-6 loaded NLCs; (A) Control cells with DAPI; (B) Control cells with ActinRed 555 (C) Control cells with coumarin-6; (D) Merged image of control cells; (E) Cells treated with coumarin 6-loaded NLCs, with DAPI; (F) Cells treated with coumarin 6-loaded NLCs, with ActinRed 555; (G) Cells treated with coumarin 6-loaded NLCs; (H) Merged image of cells treated with coumarin-loaded NLCs. (I) Cells treated with cationic coumarin 6-loaded NLCs, with DAPI; (J) Cells treated with cationic coumarin 6-loaded NLCs, with ActinRed 555; (K) Cells treated with cationic coumarin 6-loaded NLCs: (L) Merged image of cells treated with cationic coumarin-loaded NLCs;From image G and K, it can be confirmed that the cells uptake the nanoparticles efficiently.

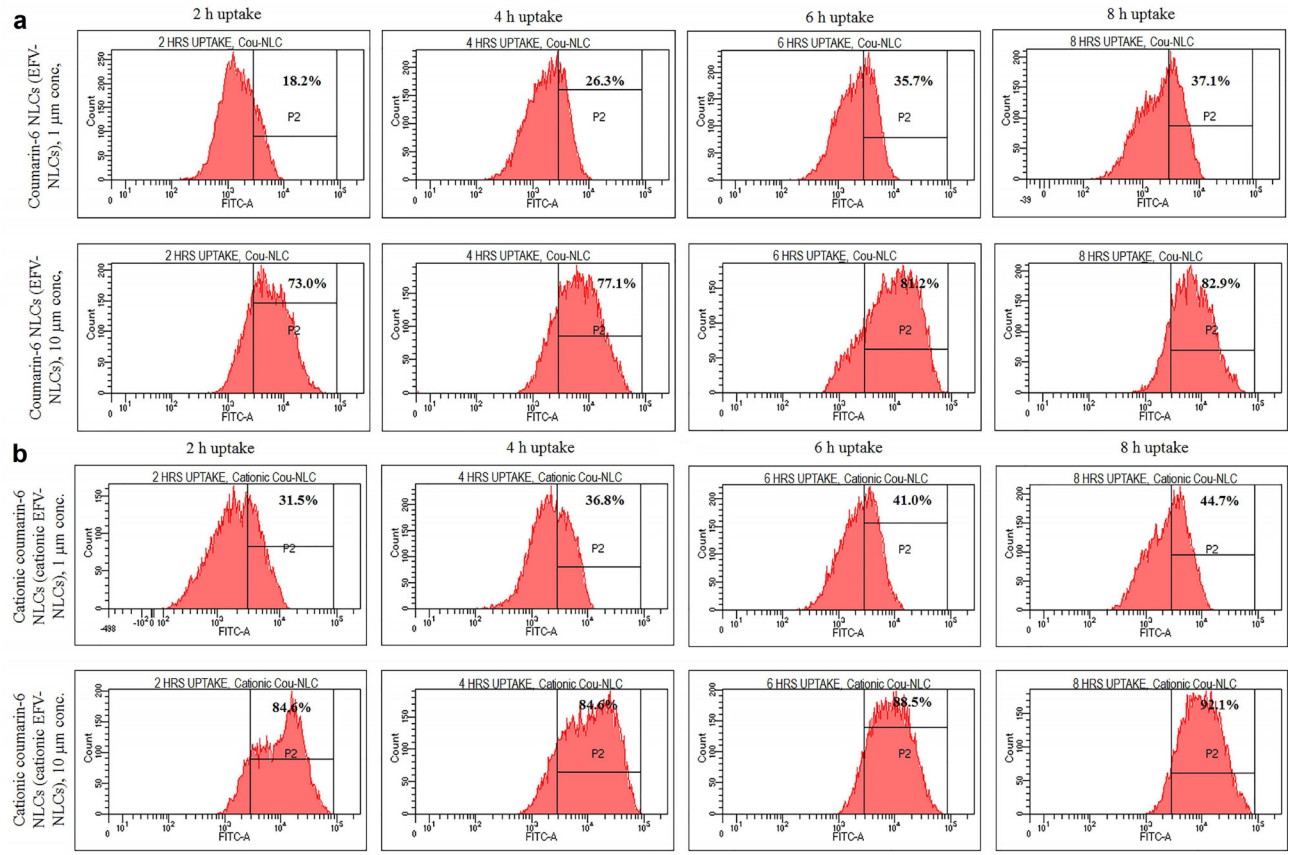

Figure 8. a) Uptake studies of coumarin-6 NLCs by flow cytometry in THP-1 cells, b) Uptake studies of cationic coumarin-6 NLCs by flow cytometry in THP-1 cells.

extent of uptake. The data presented does not represent the number or quantity of nanoparticles internalized, as the process adopted here; analyses intact cells which contain intracellular as well as surface-bound nanoparticles. It was observed that, there is a concentration dependant increase in the percentage of the cells positive for coumarin-6 loaded NLCs, suggesting that more concentration of nanoparticles will result in more concentration of drug inside the cells. In the case of coumarin 6-loaded NLCs (Figure 8a), as the concentration increased from 1 µM to 10 µM there was 2.23-fold increase i.e., from 37.1% to 82.9%; in the percentage of the cells positive for coumarin-6 loaded nanoformulations after 8 h of the treatment. A similar pattern was observed with coumarin-6 loaded cationic NLCs where uptake increased from 44.7 % to 92.1 % for 1 µM and 10 µM concentrations respectively after 8 h, which corresponded to 2.06-fold increase in the uptake. As compared to coumarin-6 loaded NLCs, cationic coumarin 6-loaded NLCs showed 1.15 to 1.73-fold higher uptake in 2 h. This is due to the presence of cationic charge present, which improves their affinity towards negatively charged cell membrane thereby facilitating cell membrane-nanoparticle interaction. We observed that after 6 h there is not much increase in the uptake of the NLCs and the uptake attains constant levels. This suggests that THP-1 cells rapidly uptake the prepared NLCs confirming their efficient uptake.

Surface charge of nanoparticles (cationic, anionic, zwitterionic) plays a vital role in cellular uptake, and previous studies suggest that zwitterionic nanoparticles enter cells via membrane fusion while cationic/anionic nanoparticles enter cells through multiple endocytic pathways.[55] Obtained results are in agreement with the proposed hypothesis that cationic nanoparticles can passively target macrophages and improve the uptake by macrophages owing to the presence of cationic charge. The results obtained also suggest that, the increase in fluorescence intensity is proportional to the time for which the cells are treated with the nanoformulations along with the concentration. The data indicates that, prepared NLCs can efficiently interact with the cells and do not harm the cells upon prolonged exposure ensuring their uptake over the period of time.

Anti-HIV efficacy by p-24 antigen assay

HIV-1 RNA, capsid antigen (p24) and anti-HIV antibody are considered as markers for the diagnosis of HIV-AIDS.[56] The levels of HIV-p24 antigen are significantly high during the early, acute and terminal phases of infection. Hence, it is considered as a helpful marker for CD4 prognosis and disease progression.[57]

To evaluate the anti-HIV efficacy of the prepared NLCs, HIV-1 p24 Antigen Capture Assay; was performed. The results are presented in percent inhibition as well as

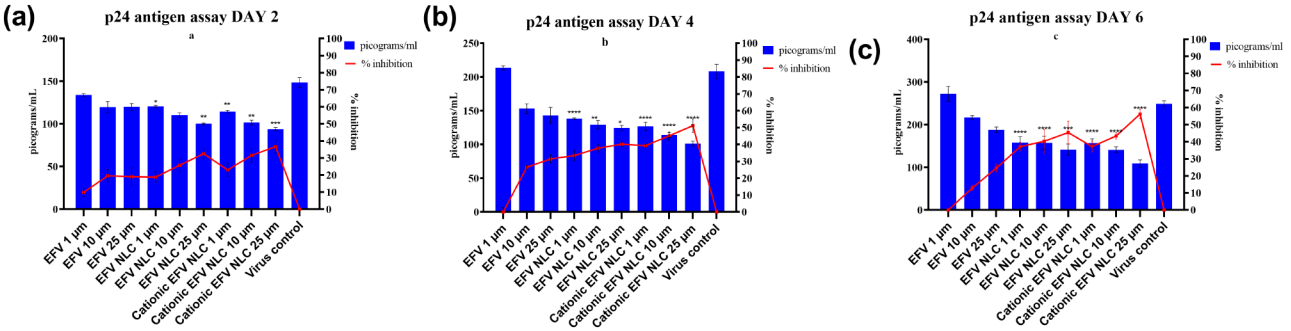

Figure 9. a) Anti-HIV efficacy studies by p24 antigen assay, Day 2; b) Anti-HIV efficacy studies by p24 antigen assay, Day 4; c) Anti-HIV efficacy studies by p24 antigen assay, Day 6.

HIV-p24 antigen levels (picograms/mL, pg/mL). Before the evaluation of anti-HIV efficacy, cytotoxicity of the prepared NLCs, as well as EFV, was carried out, subtoxic concentrations were selected on the basis of obtained CC_{50} values, for the screening of anti-HIV efficacy. Cells were treated with anti-HIV formulations for a total period of 6 days and the supernatants were analysed for p24 antigen levels on day 2, day 4 and day 6. It was observed that there is concentration dependant reduction in p24 antigen levels for EFV solution, EFV-NLCs and cationic EFV NLCs throughout the study duration (Figure 9a, b and c). On day 2 (Figure 9a) of the study, for EFV solution; p24 antigen levels at 1µM, 10µM and 25µM concentrations were 133.82 ± 1.7 pg/mL, 119.51 ± 6.7 pg/mL and 119.99 ± 3.8 pg/mL) respectively, with respect to untreated HIV-1 infected cultures (148.64 ± 5.65 pg/mL). In the case of both the NLCs, the levels of p24 antigen on day 2 (Figure 9a) were lower when compared to EFV solution, confirming efficient uptake and improvement in efficacy. On day 2, in case of EFV NLCs (Figure 9a) p24 antigen levels at 1µM, 10µM and 25 µM concentrations were 120.53 ± 1.1 pg/mL, 110.15 ± 2.6 pg/mL and 100.10 ± 1.7 pg/mL while for cationic EFV-NLCs (Figure 9a) they were 114.25 ± 1.6 pg/mL, 101.58 ± 2.6 and 93.87 ± 1.9 pg/mL, respectively. In terms of percent inhibition, at 25 µM; EFV solution demonstrated 19.08 ± 5.09 percent inhibition compared to 32.54 ± 2.9 ($P < 0.05$) percent and 36.70 ± 3.6 ($P < 0.05$) percent inhibition shown by EFV-NLCs and cationic EFV-NLCs, which was 1.70 -fold and 1.92-fold higher; respectively.

Our results are harmonious with the cellular uptake studies performed by flow cytometry, where it was observed that nanoparticle uptake increases over a period of time with respect to the concentration. However, on day 4 (Figure 9b) and day 6 (Figure 9c) of the study, it was observed that at 1µM concentration EFV solution completely fails to inhibit any HIV-1 p24 antigen levels (p24 antigen levels 213.46 ± 3.18 pg/mL on day 2 and 272.28 pg/mL on day 4, when p24 antigen level for untreated HIV-1 infected cultures was 208.46 ± 10.46 and 249.07 ± 6.61, respectively) and at higher concentrations of (10 µM and 25 µM), percent inhibition corresponded to 26.56 ± 1.07 percent and 31.47 ± 2.46 percent on day 4; and 12.95 ± 1.2 percent and 24.54 ± 2.2 percent, on day 6. From obtained data, it can be observed that EFV as a drug alone in solution form, fails to inhibit viral replication effectively, even at higher concentrations. In contrast, EFV-NLCs and cationic EFV-NLCs treated cell cultures showed lower HIV-1 p24 antigen levels consistently on day 2, day 4 and day 6 of the treatment (Figure 9a, b, c). Percent inhibition levels for NLCs were significantly higher ($P < 0.05$) on each day of the treatment as compared to EFV solution for the entire concentration range (1µM, 10 µM and 25 µM). Compared to EFV-NLCs, cationic EFV-NLCs demonstrated higher levels of percent inhibition. On the day 4 of the treatment, at 10 µM and 25 µM cationic NLCs demonstrated 45.18 ± 2.3 and 51.16 ± 4.06 percent inhibition, which was significantly higher than EFV-NLCs ($P < 0.05$). As compared to EFV-NLCs, cationic-EFV NLCs showed 1.12-fold (32.54 ± 2.92 % vs 36.70 ± 3.67 on day 2), 1.27-fold (40.19 ± 1.49 % vs 51.16 ± 4.06 % on day 4) and 1.23-fold (45.39 ± 6.74 % vs 56.21 ± 2.74 % on day 6) higher inhibition at 25 µM concentration. The percent inhibition obtained at 25 µM concentration by the cationic-EFV-NLCs was 2.29-fold higher than that of EFV alone (56.21 ± 2.74 percent vs 24.54 ± 2.23 percent). This observation indicated the capability of NLCs to release the drug in a sustained manner and revealed higher uptake as well as retention inside cells as compared to the free EFV solution. Upon encapsulation in NLCs, anti-HIV efficacy of EFV improves drastically. Interestingly, cationic EFV-NLCs outperform EFV-NLCs and EFV solution on each day of the study, emphasizing the superiority of cationic NLCs over EFV-NLCs.

In monocyte-derived macrophages, autophagy enhances the retention of nanoformulations within the cells allowing them to form macrophage depots promoting the sustained release of drug, resulting in enhanced anti-HIV activity.[58] The non-specific interactions amongst macrophage and nanoparticles are governed by particle size, shape, surface charge and hydrophobicity. Because of the presence of a positive surface charge, cationic nanoparticles are internalized more effectively by the cells. Together, this contributed to improved efficacy and better inhibition of viral replication in HIV-infected macrophages, when treated with cationic EFV-NLCs.

Conclusion

Owing to inherent fallouts of anti-HIV drugs and complexity

associated with HIV-AIDS infections, researchers have already emphasized the importance of the availability of parenteral anti-HIV therapy. However, developing parenteral nanoformulations needs to be cost-effective and shouldn't prove as an obstacle in their development. Moreover, these formulations should be able to eliminate the virus from one of the most important cellular reservoir sites, the macrophage. On this background, the cationic NLCs might represent an appealing tool for drug delivery in the treatment of HIV-AIDS. Anti-HIV drugs such as efavirenz, have higher doses and are highly lipophilic. Considering this issue, the higher encapsulation and improved solubility provided by the NLCs for anti-HIV drugs may prove to be advantageous. Cationic NLCs have advantage of better uptake by the cells due to their improved interaction with the cellular membrane. Thus, the proposed strategy of passively targeting macrophage in HIV-AIDS using cationic NLCs can prove to be fruitful to eliminate the virus. NLCs provide several other advantages such as they are stable, scalable and cost-effective, while electrostatic interactions between negatively charged macrophages and positively charged NLCs improve the cellular uptake keeping it simple to formulate without the use of complex processes or specialized chemicals/targeting ligands. Moreover, cationic EFV-NLCs demonstrated "sustained, consistent and higher" p24 antigen inhibition up to 6 days. Our studies led to the conclusion that targeting macrophages, in HIV-AIDS infection with the help of cationic NLCs resulted in a 2.29-fold increase in the anti-HIV-efficacy of EFV (NNRTI). On this basis, future studies can be designed to further explore formulating parenteral nanoformulations encapsulating one or more anti-HIV drugs to improve the therapeutic outcome.

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Authors Contributions

KM: Formulation design and development, analytical method development, *in-vitro* characterization studies, advanced characterization and optimization of nanostructured lipid carries, SR: Pre-formulation studies and optimization of formulation parameters for the development of nanostructured lipid carriers, DD: Designing cell-based assays and protocols for evaluation of anti-HIV efficacy of nanostructured lipid carriers, virus stock development and, experimental designing and optimization for cell-based assays, SK: Guidance, monitoring and approving experimental designs of nanostructured lipid carriers -based assays for anti-viral studies, PV: Guidance, monitoring pre-formulation and formulation development of nanostructured lipid carriers. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

Authors declare no conflict of interest.

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