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Intracellular delivery of redox cycler-doxorubicin to the mitochondria of cancer cell by folate receptor targeted mitocancerotropic liposomes

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ABSTRACT

Cancer cells reflect higher level of ROS in comparison to the normal cell, so they become more vulnerable to further oxidative stress induced by exogenous ROS-generating agents. Through this a novel therapeutic strategy has evolved, which involves the delivery of redox cycler-doxorubicin (DOX) to the mitochondria of cancer cell where it acts as a source of exogenous ROS production. The purpose of this study is to develop a liposomal preparation which exhibits a propensity to selectively target cancer cell along with the potential of delivering drug to mitochondria of cell. We have rendered liposomes mitocancerotropic (FA-MTLs) by their surface modification with dual ligands, folic acid (FA) for cancer cell targeting and triphenylphosphonium (TPP) cations for mitochondria targeting. The cytotoxicity, ROS production and cell uptake of doxorubicin loaded liposomes were evaluated in FR (+) KB cells and found to be increased considerably with FA-MTLs in comparison to folic acid appended, mitochondria targeted and non-targeted liposomes. As confirmed by confocal microscopy, the STPP appended liposomes delivered DOX to mitochondria of cancer cell and also showed higher ROS production and cytotoxicity in comparison to folic acid appended and non-targeted liposomes. Most importantly, mitocancerotropic liposomes showed superior activity over mitochondria targeted liposomes which confirm the synergistic effect imparted by the presence of dual ligands – folic acid and TPP on the enhancement of cellular and mitochondrial delivery of doxorubicin in KB cells.

1. Introduction

Improving therapeutic efficacy and selectivity is a major challenge in the development of anticancer therapeutics. So a novel approach of target-specific drug delivery might be required to effectively eliminate these cells. Mounting evidence suggests that, compared with their normal counterparts, many types of cancer cells have increased levels of reactive oxygen species (ROS) (Szatrowski and Nathan, 1991; Kawanishi et al., 2006). A moderate increase in ROS can promote cell proliferation and differentiation, whereas excessive amounts of ROS can cause oxidative damage to lipids, proteins and DNA. Therefore, maintaining ROS homeostasis is crucial for normal cell growth and survival. An increase in ROS is associated with abnormal cancer cell growth and reflects a disruption of redox homeostasis either due to an elevation of ROS production or to a decline of ROS-scavenging capacity, a condition known as oxidative stress (Cadenas and Davies, 2000). Moreover, cancer cells with increased oxidative stress are likely to be more vulnerable to damage by further ROS insults induced by exogenous agents (Pelicano et al., 2004). Therefore, manipulating ROS levels by redox modulation is a way to selectively kill cancer cells without causing significant toxicity to normal cells (Schumacker, 2006). It is believed that these malignant cells would be more dependent on antioxidants for cell survival and, therefore, more vulnerable to further oxidative insults induced by ROS-generating agents known as 'Redox Cyclers' (Trachootham et al., 2009). Doxorubicin is an example of redox cycler that forms a radical intermediate which may react with flavoprotein reductases such as cytochrome P450 reductase and NAD(P)H:quinineoxidoreductase (NQO1) in the presence of reduced NADPH. These derivatives generate superoxide in the presence of molecular oxygen. Another mechanism of doxorubicin-induced ROS production is the intracellular chelation of iron, which may trigger a Fenton-type reaction leading to the generation of the highly reactive hydroxyl radical (Myers et al., 1977). Mitochondria

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as a subcellular target for DOX have been thoroughly reviewed by Jung and Reszka (2001).

During the last decade, intracellular drug delivery has become an emerging area of research in the medical and pharmaceutical fields. Among all other intracellular sites mitochondria seems to be a plausible drug delivery target as it makes an integral contribution in the regulation of several aspects of cell biology such as energy production, molecular metabolism, redox status, calcium signaling and programmed cell death. Moreover, properties of mtDNA make it an attractive target for chemical attack: a lack of protective histones renders it almost 500 times more sensitive than nuclear DNA, a condition that is further exacerbated by its limited capacity for repair (Preston et al., 2001). The intense research in the field of anticancer therapy signifies that for achieving optimal therapeutic effect an administered drug or a bioactive molecule/macromolecule must safely reach not only its target cell but also to the appropriate location within that cell (Moghimi and Rajabi-Siahboomi, 2000; Langer, 1998). To date, numerous investigators have attempted to construct mitochondria-targeted delivery systems; however, it is still difficult to efficiently deliver therapeutic drugs to mitochondria of cancer cell (Mukhopadhyay and Weiner, 2007; Yamada et al., 2007). For the delivery of drugs to mitochondria, Boddapati et al. (2005) rendered plain liposomes mitochondriotropic by the anchoring of triphenylphosphonium cations to the liposomal phospholipid bilayer. The triphenylphosphine (TPP) is a cationic lipophilic compound which has been reported to be an ideal ligand for mitochondrial targeting (Murphy, 2008). However, the cationic liposomes have tendency to aggregate with serum proteins or blood cells (Li et al., 1999) and adhere electrostatically to vascular endothelium cells after intravenous injection, resulting in their rapid clearance from the bloodstream or their localization in the lungs with the risk of causing lung embolisms (Simberg et al., 2003). In addition, cationic liposomes are also associated with initiation of inflammatory reactions and activation of serum complement system (Zelphati et al., 1998). These potential limitations, however, is the propensity of cationic liposomes to be rapidly eliminated from circulation by the "first-pass" organs, such as the lungs, the liver and the spleen. The inclusion of high-molecular-weight polymers, such as polyethylene glycol (PEG), on the liposome surface is considered as an efficient approach to limit the interaction of conventional liposomes with circulating blood proteins, blood cells or cells of MPS, and thus prolonging their blood circulation time (Ceh et al., 1997), which enables them to accumulate in tumor tissue due to the effect of enhanced permeability and retention (EPR) (Maeda et al., 2000; Harrington et al., 2001). Although this sterically stabilization of liposomes with PEG enables liposomes to accumulate in tumor tissue, it causes a reduction in liposomes interaction with the target cells (Harvie et al., 2000; Vertut-Doi et al., 1996), leading to lower uptake of the entrapped drugs via cell endocytosis or membrane fusion. In addition, it has been reported by several investigators that folate receptors are overexpressed in a wide variety of human cancer cells, especially ovarian carcinomas and present in low or non-detectable levels in most normal cells (Buist et al., 1995; Ross et al., 1994; Wu et al., 1999). This receptor following binding to its ligand undergoes internalization by receptor mediated endocytosis, delivering its cargo into cell interior (Lee et al., 1996). This receptor has been exploited to deliver liposome entrapped anticancer cytotoxic drugs and various free therapeutic agents (Lu and Low, 2002) specifically to cancerous cells over expressing folate receptors.

In this present investigation we have developed a suitable carrier system for the cancer cell specific mitochondrial delivery of DOX. We have rendered liposomes mitocancerotropic by the attachment of dual ligands to phospholipid bilayer, folic acid attached for cancer cell targeting and TPP attached for mitochondrial targeting. Folic acid was appended on the liposomal surface with the help of the FA–PEG–Chol conjugate, where cholesterol forms an anchor for the phospholipid bilayer entanglement of folic acid. The mitochondriotropic ligand was incorporated to liposomal bilayer in the form of stearyl triphenylphosphine (STPP), where stearyl chain forms an anchor for the phospholipid bilayer entanglement of TPP. Further, we have explored the potential of mitocancerotropic liposome (FA-MTLs) preparation in delivering the redox cycler-DOX to the mitochondria of KB cells. The effect of DOX release from PEG appended cationic liposomes was examined in the presence of serum protein. We hypothesized that the incorporation of DOX into mitocancerotropic liposomes (surface decorated with folic acid, STPP and PEG) could improve its cytotoxic action over other DOX loaded non-targeted (NTLs), folic acid conjugated (FALs) and mitochondriotropic (MTLs) liposomes, with further attempt of decreasing DOX dose to achieve higher therapeutic effect. The cellular uptake, intracellular distribution and ROS production from different liposomal formulation was also investigated.

2. Materials and methods

2.1. Materials

Doxorubicin hydrochloride (DOX) was obtained as a gift sample from Sun Pharmaceuticals, Halol (India). The egg phosphatidyl choline (EPC), cholesterol (Chol), triphenylphosphine (TPP), sephadex G-15 & G-50, dialysis tubing, folic acid, N-hydroxysuccinimide (NHS), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), polyoxyethylene bis-amine (MW, 3350, NH_2–PEG–NH_2), cholesteryl chloroformate, stearyl bromide, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT), fetal bovine serum (FBS), penicillin–streptomycin, paraformaldehyde and folate-free RPMI 1640 media were purchased from Sigma–Aldrich Chemical Co. (Mumbai, India). Hoechst 33342 and MitoFluor Green were purchased from Invitrogen Corporation, New York, USA. All other reagents and solvents were of analytical or HPLC grade and used without further purification.

2.2. Synthesis of mitochondriotropic ligand (STPP)

The mitochondriotropic residues, such as cationic groups (triphenyl phosphine) were attached to the liposomal bilayer to achieve mitochondrial delivery of liposomes. Stearyl triphenylphosphonium bromide was synthesized following the protocol of Boddapati et al. (2005) with modification in purification protocol. For liposomal incorporation triphenylphosphine was linked to stearyl residue. Briefly, stearyl bromide (1.93 g) and triphenylphosphine (1.52 g) were heated under reflux in anhydrous xylene (30 ml) for the period of 24 h. The progress of reaction was monitored by thin layer chromatography and the crude obtained after solvent removal was purified employing silica gel chromatography using methanol:ethyl acetate (5:95) as an eluent. The purified STPP obtained was crystallized from diethyl ether and was characterized using ^{31}P NMR, ^{1}H NMR spectroscopy and thin layer chromatography.

2.3. Synthesis of cancerotropic ligand (folic acid–PEG–cholesterol)

The synthesis was carried out as shown in Fig. 1. The conjugation of folic acid–PEG–cholesterol (FA–PEG–Chol) was done in two steps with some modification in process described by Lee et al. Firstly, folic acid was conjugated with bis-amine PEG to yield

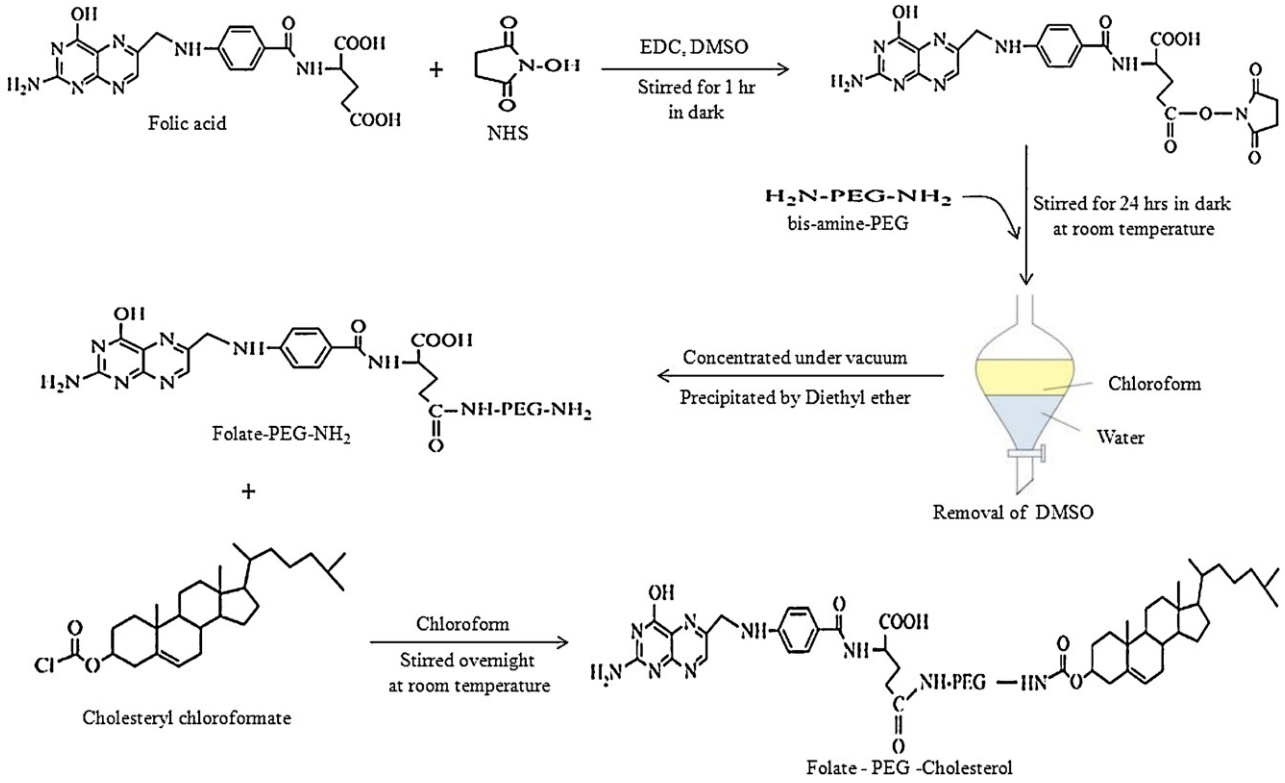

Fig. 1. Synthesis pathway for FA–PEG–Cholesterol.

FA–PEG–NH_2 which was further conjugated to cholesteryl chloroformate in second step to yield FA–PEG–Chol (Guo et al., 2000). Briefly, folic acid was dissolved with excess of DMSO along with 1.1 M excess of EDC and 1.0 M equivalent of NHS. The reaction mixture was stirred for 1 h in dark and then 1.0 M equivalent of bis-amine-PEG was added to reaction mixture and stirred for 24 h in dark at room temperature. The progress of reaction was monitored through thin layer chromatography and by monitoring the depletion of primary amines measured by a ninhydrin assay, considering conversion of 50% initial amines as reaction end point. The FA–PEG–NH_2 was extracted in chloroform layer by three consequent washing of reaction mixture with chloroform/water (1:1, w/w). To remove the traces of water present in separated chloroform layer, it was treated with anhydrous sodium sulphate and was allowed to precipitate by the addition of non-solvent, diethyl ether. Further, FA–PEG–NH_2 was treated with 1.1 molar excess of cholesteryl chloroformate in chloroform and reaction was carried out overnight at room temperature. Progression of reaction was monitored by complete disappearance of free amino group using ninhyrin assay. The product FA–PEG–Chol was precipitated by the addition of diethyl ether and was washed twice with ether to remove any residual cholesteryl chloroformate. Both FA–PEG–NH_2 and FA–PEG–Chol were characterized by thin layer chromatography and ^{1}H NMR using DMSO as a solvent on Bruker Avance II 400 MHz NMR spectrometer.

2.4. Preparation of liposomes and DOX loading

Liposomes were prepared using thin film hydration method followed by sonication to reduce vesicle size (Gabizon and Papahadjopoulos, 1988) and then loaded with DOX using ammonium sulphate gradient driven loading technique (Gabizon et al., 2003). The lipid composition of prepared liposomes were as follow: (1) non-targeted liposomes (NTLs), EPC:Chol = 12.9:6.0 (molar ratio, total lipid content 20 mg/ml); (2) cancerotropic liposomes (FALs) – folic acid conjugated liposomes, EPC:Chol:FA–PEG–Chol = 12.9:6.0:0.2 (molar ratio, total lipid content 20 mg/ml); (3) mitochondriotropic liposomes (MTLs) – STPP appended liposomes, EPC:Chol:STPP, 12.9:6.0:6.0 (molar ratio, total lipid content 20 mg/ml) and (4) mitocancerotropic liposomes (FA-MTLs) – with both FA–PEG–Chol and STPP incorporated to liposomal bilayer, EPC:Chol:STPP:FA–PEG–Chol = 12.9:6.0:6.0:0.2 (molar ratio, total lipid content 20 mg/ml). Briefly, a required amount of lipids EPC, Chol, STPP and FA–PEG–Chol in desired molar ratios were dissolved in chloroform. The thin lipid film was formed by removing chloroform under the influence of vacuum and heating using laborota (Heidolph, Germany). Formed lipid film was then hydrated with ammonium sulphate solution (250 mM) using 4 mm glass beads to produce crude suspension that was further subjected to sonication at 60 amplitude for 5 min (temperature during process was between 40 and 50 °C) using probe sonicator (Ultrasonic liquid processor, Misonix, USA). The resultant liposomal preparation was centrifuged at 3000 rpm for 3 min at 25 °C to remove the titanium particles possibly shed from probe.

The liposomes prepared were further processed for buffer exchange cycles against HEPES buffer (pH 7.4) to remove extra liposomal ammonium sulphate using tangential flow filteration (TFF) technique employing 'Biomax' polyethersulphone (100 kDa, cut off membrane) cassette. The liposomal preparation was first allowed to concentrate upto 25 ml and than volume of reservoir was kept constant by continious addition of HEPES buffer (pH-7.4) until 250–300 ml of permeate is collected. The resulted liposomal suspension was further incubated with DOX (2 mg/ml) at 60 °C for 1 h in shaking incubator to achieve loading.

2.5. Morphology and zeta-potential of liposomes

In order to determine effect of DOX loading process on liposomes, particle size and polydispersity index (PDI) was determined

both before and after loading of liposomes using Submicron Particle Size Analyzer (Delsa™ Nano C) instrument by Beckman Coulter, Inc. For morphological characterization and particle size determination of liposomes, transmission electron microscopy (TEM) was done using Hitachi S-7500 transmission electron microscope (Hitachi, Japan). Diluted drop of liposomal dispersion was placed on a 200-mesh carbon coated copper grid. The photographs were taken at 40,000× magnification and 80 kV voltages (Reddy and Murthy, 2004).

Zeta potential were measured using electrophoretic light scattering (ELS), which determines electrophoretic movement of charged liposomes under an applied electric field from the Doppler shift of scattered light, for zeta potential determination. The zeta potential of concentrated liposomal suspension was determined using forward scattering through transparent electrode technology (FST) without any further dilution.

2.6. Liposomal incorporation of STPP and folic acid

The liposomes (MTLs and FA-MTLs) prepared by incorporation of STPP were separated from non-incorporated STPP using gel permeation chromatography on a Sephadex G-15 column. The separated liposomes were lyophilized and were characterized using ^{31}P NMR spectroscopy. For confirming liposomal incorporation of folic acid, the above separated liposomal suspension was dissolved in 90% isopropanol containing 10% (0.075 mol/L) HCl and folate–PEG–Chol content was determined by measuring the folate content in the product using UV extinction at 363 nm.

2.7. DOX encapsulation efficiency

The unentrapped DOX was removed from liposomes using gel permeation chromatography employing sephadex G-50 as sationary phase. The filtrate collected was dissolved in 90% isopropanol containing 10% (0.075 mol/L) HCl and DOX content was determined spectrometrically at 480 nm. The encapsulation efficiency of DOX is represented using the following equation (Niu et al., 2010).

$$\text{encapsulation efficiency } (E.E\%) \text{ of DOX} = \frac{A_1 \times V_1}{A_0} \times 100$$

where A_1 is absorbance of filtrate collected from column and A_0 is absorbance of unfiltered liposomal dispersion. V_1 refers to the volume obtained after running of liposomal sample from column.

2.8. In vitro drug release

In vitro drug release of liposomes was observed in phosphate buffer saline (PBS, pH 7.4) with and without 10% (w/v) bovine serum albumin (BSA). Dialysis membrane with cut off 12 kDa was filled with 1 ml of liposomal suspension and sealed from both ends. The bag was placed in 50 ml round bottom flask filled with 50 ml of PBS (with and without 10%, w/v BSA), each sample in triplicate. The flask was allowed to shake at 100 rpm at 37 °C in shaking incubator. At predetermined time interval, 1 ml aliquot aqueous solution was withdrawn from flask and replaced with fresh buffer to maintain non-saturated sink conditions. The amount of doxorubicin released in each time interval was determined using UV–vis spectrophotometer as described elsewhere in the text.

2.9. Cell culture and maintenance

Human oral carcinoma KB cell line was purchased from NCCS (Pune, India) and cultured in folate-free RPMI 1640 media, supplemented with 50 μg/mL penicillin, 50 μg/mL streptomycin and 10% fetal bovine serum (FBS) and maintained in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.10. Cell uptake studies of liposomes by flow cytometry

Intracellular uptake of liposomes was determined by flow cytometry assay (Hwang et al., 2007). Briefly, the KB cells were transferred to 24-well tissue culture plates at 1×10^4 cells per each well and incubated for 12 h at 37 °C. The culture medium was replaced with different liposomal formulations (equivalent to 20 μg of DOX) and then incubated for 2 h at 37 °C in medium. The culture medium was then removed and each well was washed with cold phosphate buffered saline (PBS, pH 7.4) solution and 300 μl of paraformaldehyde (5%, v/v) was added to each well to fix the cells. The samples were analyzed using a FACS-Calibur™ flow cytometer, BD Biosciences. Cell-associated DOX was excited with an argon laser (488 nm) and fluorescence was detected at 560 nm. Data were collected of 10,000 gated events and analyzed with the CELL Quest software program.

To confirm the folate receptor mediated uptake of liposomes, folate receptors on cell surface were blocked by the addition of 1 mM of free folate to the incubation media (Li et al., 2010). KB cells suspension was further incubated with Dox-FALs and Dox-FA-MTLs for 2 h at 37 °C. The culture media was washed, fixed and analyzed by flow cytometry as per the protocol discussed above.

2.11. Intracellular distribution studies

For intracellular distribution study of liposomes we used confocal laser scanning microscopy (Akita et al., 2004). The KB cells were seeded on glass cover slips (18 mm × 18 mm) placed into 35 mm tissue culture plates at a density of 2×10^5 cells (in 2 mL growth medium). After overnight growth, supernatants from the culture plates were aspirated out. Further, cells were exposed to fresh aliquots of growth medium containing DOX loaded liposomal formulations (including Dox-FALs, Dox-MTLs and Dox-FA-MTLs) for the period of 2 h. Following incubation the cells were washed with phosphate buffered saline (PBS) and stained with MitoFluor Green (Molecular Probes/Invitrogen) for 30 min and Hoechst 33342 (Molecular Probes/Invitrogen) as recommended by the manufacturer. The cells were washed with PBS and mounted in Fluoromount G medium. Fluorescence micrographs of the blue, red and green channels within the same field were overlaid and co-localization of red fluorescence of DOX with green fluorescence of stained mitochondria and with blue fluorescence of stained nucleus was observed under Zeiss Meta 510 LSM with Zeiss software.

2.12. Evaluation of intracellular ROS production

The formation of reactive oxygen species was evaluated by means of the probe 2,7-dichlorodihydrofluorescein diacetate (H2DCF-DA), which is a non-fluorescent permanent molecule that passively diffuses into cells and gets converted to H2DCF by the cleavage of acetate by intracellular esterases resulting in its entrapment within the cell. In the presence of intracellular ROS, H2DCF get rapidly oxidized to the highly fluorescent 2,7-dichlorofluorescein (DCF) and thus measured through florescence spectroscopy (Wang and Joseph, 1999). Briefly, KB cells at 2×10^4/well were seeded into 96-well plate and allowed to attach for 24 h. Then, the wells were replaced with fresh medium and treated with DOX loaded NTLs, FALs, MTLs and FA-MTLs equivalent to 20 μg per well concentration of DOX. DOX unloaded NTLs, FALs, MTLs and FA-MTLs were taken as respective control (to evaluate the effect of formulation components on ROS production) and were incubated for 4 h at 37 °C in CO_2 incubator. After 4 h, supernatant were aspirated out and the cells were washed twice with fresh growth medium to ensure complete removal of DOX and liposomes present outside the cells and the cells were further incubated upto 24 h at 37 °C. After treatment, the medium was removed and the cells were washed thrice with

PBS (pH 7.4). Then 2,7-dichlorodihydrofluorescein diacetate was added at a final concentration of 10 μM and incubated for 1 h at 37 °C. After 1 h of incubation, fluorescence was monitored at an excitation wavelength of 502 nm and an emission wavelength of 520 nm using Envision 2104 Multilabel Reader (PerkinElmer Life Sci., USA).

To eliminate the possible fluorescence emitted by DOX, a separate experiment was conducted using a negative control for each liposomal formulation by following the above mentioned protocol with some modification. The fluorescence was monitored at an excitation wavelength of 502 nm and an emission wavelength of 520 nm, without the addition of 2,7-dichlorodihydrofluorescein diacetate to the wells. In brief, the cells (2×10^4/well) were seeded in 96-well plate and allowed to attach for 24 h. Then, the wells were replaced with fresh medium and treated with DOX loaded NTLs, FALs, MTLs and FA-MTLs equivalent to 20 μg DOX per well using DOX unloaded NTLs, FALs, MTLs and FA-MTLs as respective controls and incubated for 4 h at 37 °C. Similarly, after 4 h of incubation cell were washed with fresh medium and incubated further for up to 24 h at 37 °C. After 24 h of treatment, the medium was removed and the cells were washed thrice with PBS (pH 7.4) and the fluorescence was determined.

Results were expressed as percentage increase in fluorescence per well using formula:

$$\text{percentage fluorescence increase} = \frac{[\{\mathbf{F}(\text{DCF})_{\text{treated}} - \mathbf{F}(\text{DCF})_{\text{untreated}}\} - \{\mathbf{F}(\text{without DCF})_{\text{treated}} - \mathbf{F}(\text{without DCF})_{\text{untreated}}\}]}{\mathbf{F}(\text{without DCF})_{\text{untreated}}} \times 100$$

where **F**(DCF) and **F**(without DCF) are fluorescence of KB cells measured after treatment of 24 h incubated cells with and without addition of 2,7-dichlorodihydrofluorescein diacetate, respectively. The group treated and untreated signifies, the cells treated with DOX loaded liposomal formulation and cells treated with DOX unloaded liposomal formulation, respectively.

2.13. Comparative cytotoxicity studies of DOX loaded liposomes

Cell viability was tested using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Gerlier and Thomasset, 1986) based on the cleavage of yellow tetrazolium salt MTT by metabolically active cells to form an orange formazan dye which was quantified using ELISA reader (Biorad, USA, model 680). Cells were seeded in 96 well microliter plates at 2×10^4 cells per well, followed by overnight incubation. Supernatants from the wells were aspirated out and replaced with fresh aliquots of growth medium containing different concentration of Dox-NTLs, Dox-FALs, Dox-MTLs and Dox-FA-MTLs (equivalent to 0.05–20 μg DOX/well) using NTLs, FALs, MTLs and FA-MTLs as respective controls and incubated for 4 h at 37 °. After 4 h, supernatant were aspirated out and the cells were washed twice with fresh growth medium to ensure complete removal of DOX and liposomes present outside the cells. The cells were further incubated for 24 h at 37 °. After 24 h supernatants were aspirated out and the cell monolayers in the wells were washed with 200 μL PBS (0.1 M, pH 7.4). Subsequently, MTT reagent (150 μL, 0.8 mg/mL) was added to each well, incubated for 5 h. Dimethyl sulphoxide (100 μL) was added in each well after aspirating out the supernatant and incubated at 37 °C for 1 h. Absorbance at two wavelengths (570 nm for soluble dye and 630 nm for cells) was recorded using ELISA reader. Concentrations of samples showing 50% reduction in cell viability (i.e. IC_{50} values) were then calculated. An OD value of control cells (unexposed cells) was taken as 100% viability (0% cytotoxicity).

3. Results and discussion

Previous studies reveals that STPP renders liposomes mitochondriotropic, and that such formulation are ideally suited for the delivery of poorly soluble drugs to mitochondria (Boddapati et al., 2005; Weissig et al., 2006). Whereas Lee et al. previously studied that folic acid conjugation to liposomes increases its intracellular uptake to many fold in comparison to the non-targeted liposomes. The preliminary experiments in our labs revealed that maximum encapsulation of DOX to liposomes can be achieved at EPC to cholesterol molar ratio of 12.9:6.0. In order to evaluate effect of STPP and FA–PEG–Chol incorporation to phospholipid bilayer and to achieve optimized liposomal formulation with desired characteristics we have varied their concentration by keeping EPC:Cholesterol ratio constant.

3.1. Synthesis and liposomal incorporation of STPP

The STPP was synthesized using Wittig reaction; the stearyl bromide and triphenylphosphine were heated under reflux in anhydrous xylene according to the protocol discussed in section above. Pure product was isolated by column chromatography (elution cycle presented in Fig. 2) on silica gel and was crystallized using diethyl ether as non-solvent in yield of 45–50%. Further final product was characterized using thin layer chromatography and NMR (^{31}P and ^{1}H) spectroscopy and was found to be in accordance with the previously published literature (Boddapati et al., 2005). The NMR interpretation are: ^{1}H NMR ($CDCl_3$, 300 MHz, δ, TMS = 0) 7.68–7.78 (m, 15H), 3.83(m, 2H), 1.63 (m, 4H), 1.18–1.29 (m, 28H), 0.87 (t, 3H, J = 5. 1 Hz) and ^{31}P NMR δ: 24.43.

Liposomal incorporation of STPP in MTLs and FA-MTLs was confirmed from ^{31}P NMR of lyophilized liposomes. ^{31}P NMR of STPP, MTLs and FA-MTLs is presented in Fig. 3. The ^{31}P NMR of STPP incorporated liposomes showed two chemical shifts corresponding to phosphorous in lipids phosphate group (Peak I) and to the charged phosphorus of STPP (Peak II). The peak II corresponding to mitochondriotropic ligand-STPP was observed at δ: 24.55 (MTLs) and at δ: 24.31 (FA-MTLs). No change in chemical shift between free STPP and lipids in NTLs was observed in comparison to chemical shift observed in MTLs and FA-MTLs which suggest the liposomal bilayer incorporation of STPP without any interaction with the components of liposomal bilayer and FA–PEG–Chol. Moreover STPP is a cationic ligand; its incorporation to the liposomal bilayer has shifted the zeta-potential of liposomes from negative to the positive value without imparting any significant effect on the size of the liposomes. A plateau phase in zeta potential was observed after 6.0 moles of STPP incorporation in MTLs and could be correlated to the attainment of maximum incorporable limit of STPP in the EPC/Chol liposomes.

3.2. Synthesis and liposomal incorporation of FA–PEG–Cholesterol

Folic acid–PEG–Cholesterol was synthesized in two steps reaction (Fig. 1). Firstly, conjugation of folic acid and PEG-bis-amine was conducted using NHS and EDC as coupling agents. The product FA–PEG–NH_2 was extracted in chloroform layer and precipitated by addition of diethyl ether. Product characterized by ninhydrin assay showed the presence of free amine groups and thin layer chromatography confirmed the conjugation of folic acid and polyoxyethylene-bis-amine. In second step, FA–PEG–NH_2 was further conjugated with cholesteryl chloroformate as described earlier. Purity of final conjugate was confirmed using silica gel thin layer chromatography, the FA–PEG–Chol appeared as single

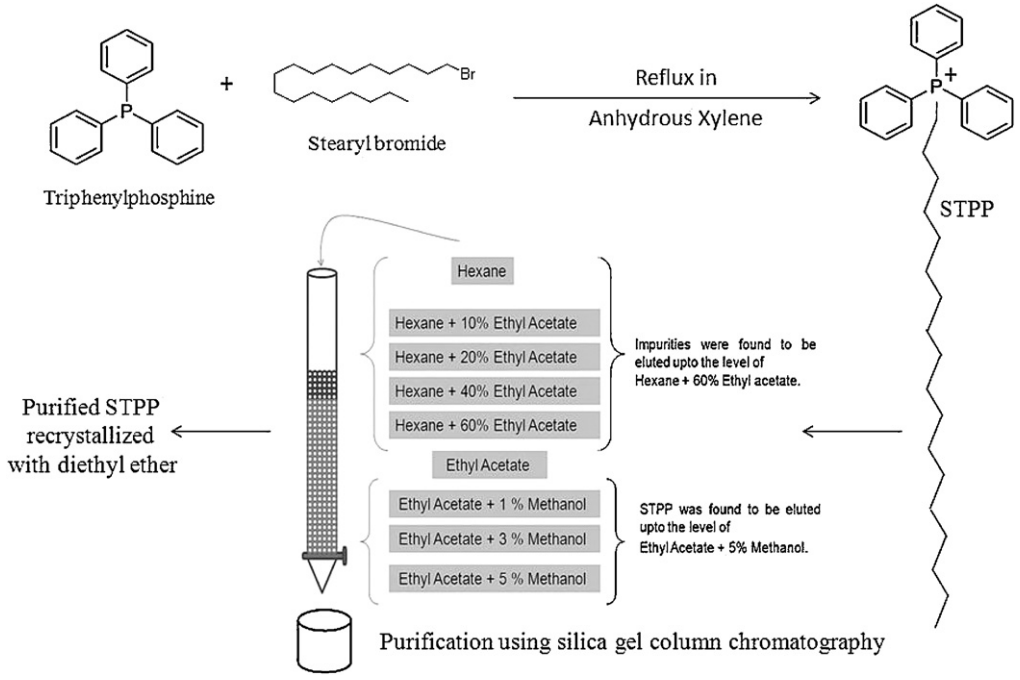

Fig. 2. Synthesis and purification pathway for Stearyltriphenyl phosphine (STPP).

spot with an R_f of 0.82 in solvent system composed of chloroform/methanol (7/3 v/v, with traces of ammonia). Fig. 4 shows the ^{1}H NMR of final conjugate of FA–PEG–Chol and confirms the formation of the conjugate. ^{1}H NMR analysis showed principal peaks (in ppm) related to folate moiety [8.71 (s), 7.62 (d), 6.66 (d), 4.49 (s)], the PEG moiety [3.22–3.72 (m)] and the cholesterol moiety [5.35 (d), 1.11 (d), 1.21 (s), 1.51–2.42 (m)].

The liposomal incorporation of FA–PEG–Chol was confirmed by the UV absorption of folate observed at 363 nm. The saturation level of folate absorbance for FA-MTLs of size ~ 150 nm was observed at 0.2 moles of FA–PEG–Chol (at total lipid content of 20 mg/ml), which confirms the maximum incorporable capacity of the lipid bilayer for FA–PEG–Chol in the presence of 6.0 moles of STPP.

3.3. Liposome characterization

Liposomes with different compositions (present in Fig. 5) were prepared by thin film hydration followed by sonication, as described in above section. The ammonium sulphate loaded liposomes were further processed for buffer exchange cycles to build trans-membrane ammonium gradient. The buffer exchanged liposomes were incubated with DOX solution for 1 h leading to entrapment of doxorubicin to liposomes. As may be noted from Table 1, all liposomal formulations showed DOX entrapment efficiency of more than 90.0%, where MTLs showed lowest entrapment efficiency of 91.0 ± 1.2% among all liposomal formulations. All liposomal formulation demonstrated a mean diameter of less than 200 nm (presented in Table 1), which make them eligible to exploit

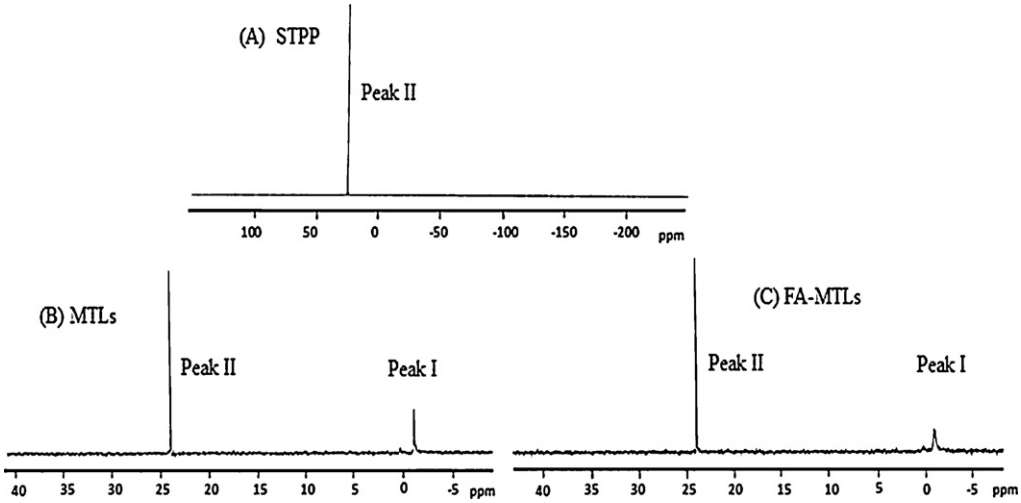

Fig. 3. ^{31}P NMR of STPP, MTLs and FA-MTLs presenting two chemical shifts corresponding to phosphorous in lipids phosphate group (Peak I) and to the charged phosphorus of STPP (Peak II), confirming the liposomal bilayer incorporation of STPP.

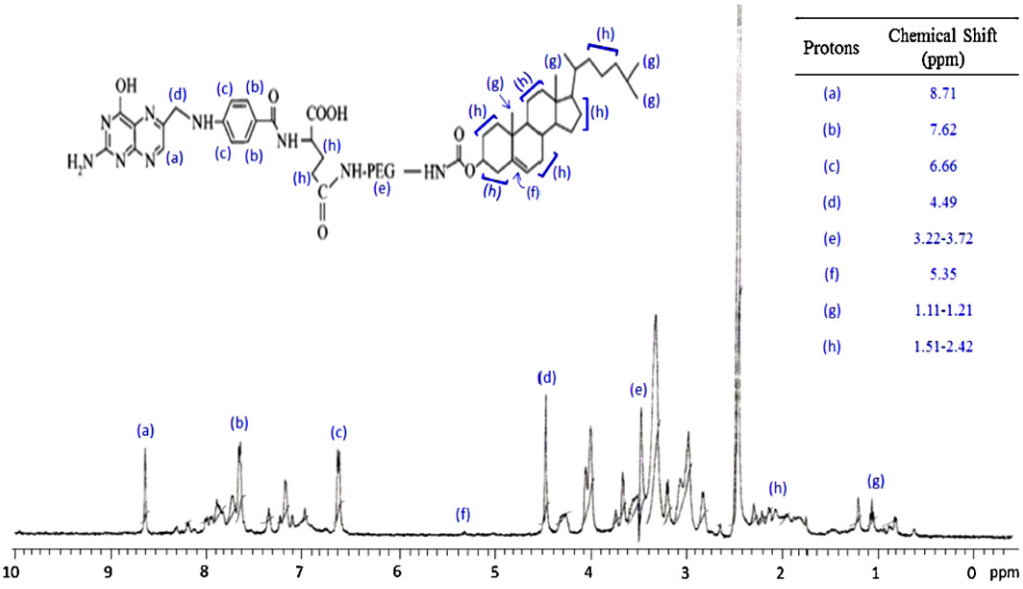

Fig. 4. Represents the ^{1}H NMR of folic acid–PEG–Cholesterol conjugate.

Table 1
Particle size, zeta potential and polydispersity index (PDI) and doxorubicin entrapment efficiency in different liposomal preparations (mean ± S.D., $n = 3$).

Formulations	Size (nm)		PDI		Zeta-potential (mV)	DOX entrapment efficiency (%)
	Before DOX loading	After DOX loading	Before DOX loading	After DOX loading		
NTLs	137.6 ± 5.93	142.46 ± 5.49	0.26 ± 0.041	0.18 ± 0.015	−20.57 ± 1.19	95.2 ± 2.3
MTLs	146.19 ± 11.37	151.1 ± 9.95	0.24 ± 0.035	0.21 ± 0.021	22.12 ± 0.30	91.0 ± 1.2
FALs	149.07 ± 10.68	157.93 ± 13.74	0.23 ± 0.036	0.19 ± 0.062	−24.20 ± 1.79	94.1 ± 2.5
FA-MTLs	142.83 ± 11.51	150.83 ± 12.45	0.30 ± 0.055	0.20 ± 0.006	6.70 ± 1.98	92.6 ± 1.5

the enhanced permeation and retention (EPR) effect of tumor vasculature for passive delivery of drug to the cancer cells (Maruyama, 2011). However, significant increase in the size of liposomes after loading of DOX was observed in all liposomal preparations. As may be noted from TEM images (Fig. 6), the small increase in vesicle size after loading of DOX may be correlated to DOX self-association or precipitation with the salts present in the interior liposomal buffer (Abraham et al., 2005). All liposomal formulations showed a significant decrease in polydispersity index (PDI) of liposomes after loading of DOX. This decrease in PDI can be attributed to the

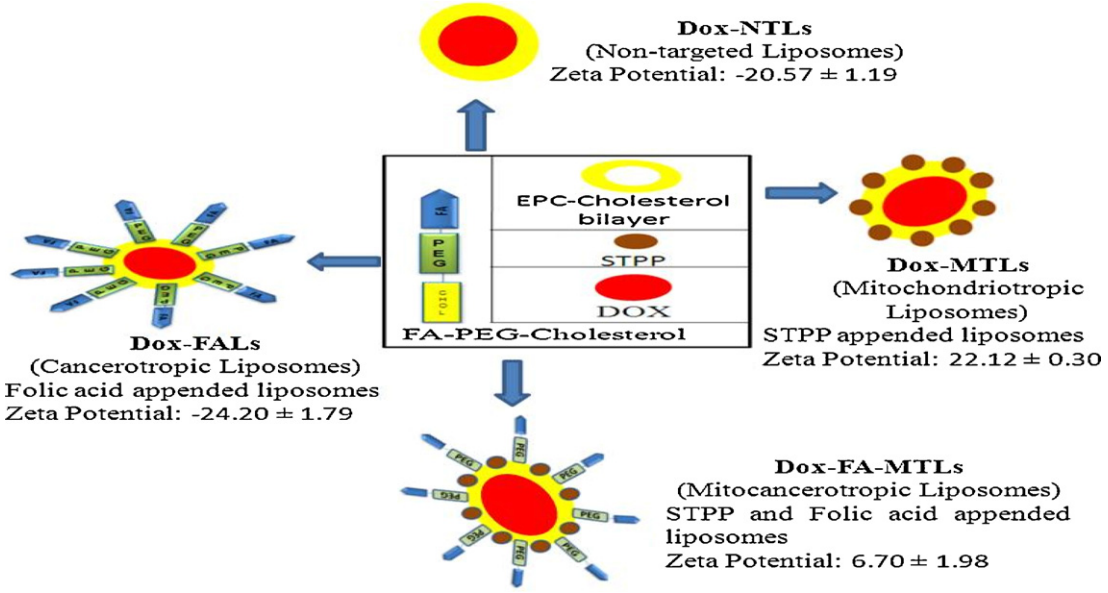

Fig. 5. Composition along with zeta-potential of different liposomes.

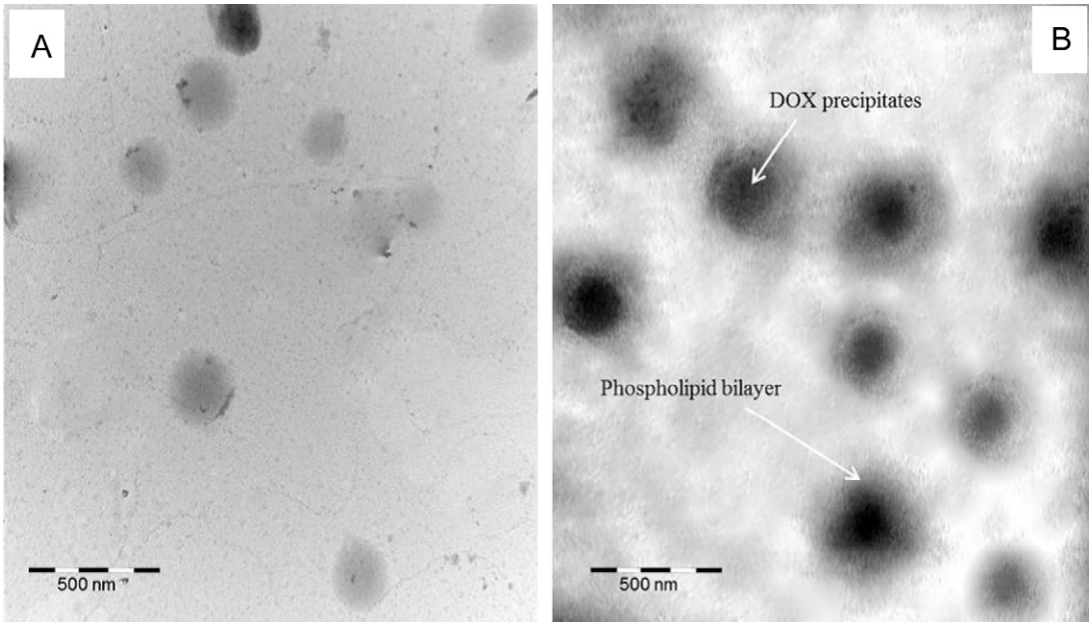

Fig. 6. Transmission electron microscopy of unloaded (A) and Dox loaded (B) FA-MTLs.

occurrence of lipids rearrangement caused by incubation of preformed liposomes at 60 °C for achieving DOX loading. Zeta potential value of NTLs was -20.57 ± 1.19 mV and this value changed to 22.12 ± 0.30 mV (for MTLS), as the result of incorporation of cationically charged STPP molecule in the phospholipid bilayer of the liposome. The zeta potential value of FALs was -24.20 ± 1.79 mV whereas; zeta potential of FA-MTLs increased to 6.70 ± 1.98 mV due to the bilayer incorporation of STPP. This increase in zeta potential toward positive value confirms the incorporation of STPP ligand in the liposomal bilayer structure.

3.4. In vitro release of liposomes

DOX release studies from liposomal preparations was conducted in PBS buffer (pH 7.4) with and without 10% w/v bovine serum albumin (BSA) at 37 ± 0.5 °C for the period of 50 h to evaluate the effect of surface modification of liposomes on DOX in vitro release profile. The curves of DOX release from Dox-NTLs, Dox-FALs, Dox-MTLs and Dox-FA-MTLs, depicts a bi-phasic drug release (Fig. 7). DOX release from liposomes involved an initial rapid release phase followed by a lag phase of relatively slow release. The cumulative percentage DOX released from Dox-NTLs, Dox-FALs, Dox-MTLs and Dox-FA-MTLs in PBS (pH 7.4,without BSA) dissolution medium after 50 h are 20.51 ± 1.1, 18.78 ± 1.9, 24.86 ± 1.37 and 22.42 ± 1.97, respectively. On the other hand, release of DOX form liposomes in the presence of serum was 33.03 ± 2.76, 27.85 ± 2.03, 39.29 ± 1.06 and 30.54 ± 1.21 respectively. In the absence of serum albumin all liposomal formulations showed sustained DOX release profile over the period of 50 h. Whereas, a significant increase in the release profile of DOX from all liposomal formulations in the presence of serum albumin was observed. The serum-induced drug leakage varied greatly with bilayer composition of the liposomes.

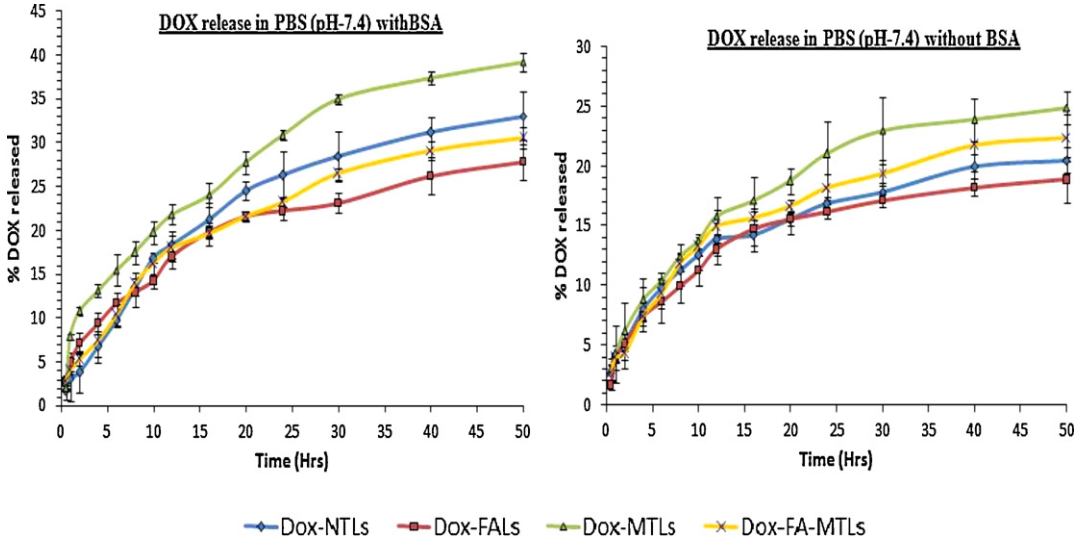

Fig. 7. Release profiles of DOX from different liposomal formulations at 37 °C in phosphate buffered saline (PBS, pH 7.4) with and without 10% w/v bovine serum albumin (BSA). Mean ± S.D. are shown ($n = 3$).

It is evident from the drug release curves that ~30% of the drug is released within 24 h in the case of Dox-MTLs, whereas other liposomal formulation showed comparative sustained release of DOX in the presence of serum albumin. The presence of cationic charges on Dox-MTLs can complex with the negatively charged serum albumin via electrostatic interaction and may have decreased the mechanical stability of the liposomal bilayer resulting in higher release of DOX. Moreover, the incorporation of cationic ligand, STPP, in the liposomal bilayer can induce gaps in membranes of lipid bilayer due to the repulsive force between STPP. The strength of liposomal membrane may tend weaker in the presence of STPP and therefore resulted in faster DOX release in comparison to other liposomal formulations. Release profile of Dox-FA-MTLs, showed a comparatively sustained response in serum albumin even in the presence of STPP. The above response can be correlated to the consideration that PEG–lipid conjugate enhances the stability of liposomes in serum by hindering absorption of serum albumin on the surface of these vesicles (Meyer et al., 1998; Stolnik et al., 1994) and hence enhanced stability of the Dox-FALs and Dox-FA-MTLs.

3.5. Intracellular uptake of liposomes evaluated by flow cytometry

To investigate the intercellular uptake of DOX transfected by liposomes, the amount of DOX uptake into the KB cells were evaluated by flow cytometry and the results are shown in Fig. 8.

Intracellular uptake (Fig. 8A) for Dox-MTLs was found to be higher than Dox-NTLs which indicate that cationic charge of MTLs can induce greater electrosatic interaction with KB cells and hence induce internalization by endocytosis (Dass, 2003; Krasnici et al., 2003). Though Dox-FALs and Dox-FA-MTLs showed higher uptake than Dox-MTLs. The uptake of these folic acid conjugated liposomes was blocked by 1 mM of free folic acid (Fig. 8B), signifying the invlovement of folate receptor mediated endocytosis of these

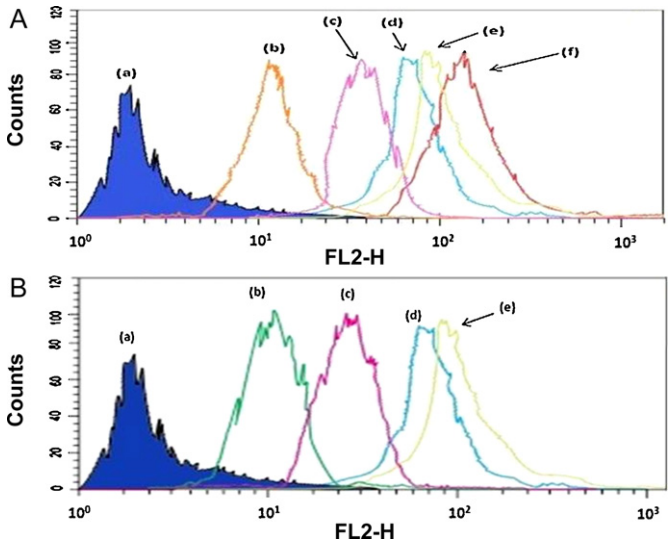

Fig. 8. Cellular uptake of DOX-loaded liposomes by flow cytometry assay. (A) represents control (a), Dox-NTLs (b), Dox-MTLs (c), Dox-FALs (d), Dox-FA-MTLs (e) and Dox-solution (f). (B) represents cellular uptake of Dox-FALs and Dox-FA-MTLs with and without the presence of 1 mM folic acid in culture medium. Control (a), Dox-FALs with (b) and without (d) the presence of folic acid (b). Dox-FA-MTLs with (c) and without (e) the presence of folic acid. Control is a background of KB cells without DOX. Mean and S.D. are shown ($n = 3$).

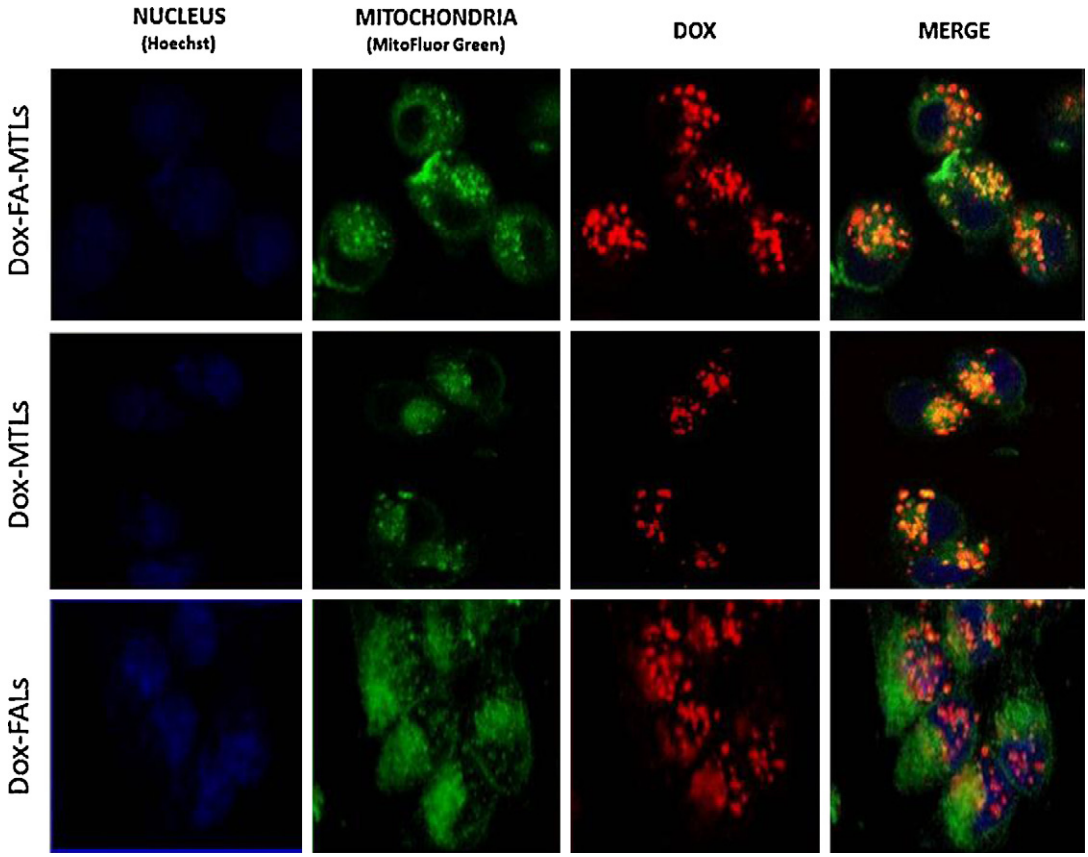

Fig. 9. Confocal fluorescence micrographs of liposomes internalization in KB cells. Green channel (EX 505 nm, EM 530 nm): MitoFluor Green stained mitochondria. Blue channel (EX 385 nm, EM 470 nm): Hoechst 33342 stained nuclei. Red channel (EX 480 nm, EM 550 nm): Doxorubicin. Yellow: represents colocalization of red and green fluorescence. Pink: represents colocalization of red and blue fluorescence.

folic acid appended liposomes. The uptake of Dox-FA-MTLs was highest among all other liposomal formulations although it was found to be lower than free DOX solution. Slightly higher uptake of Dox-FA-MTLs in comparison to Dox-FALs can be correlated to the cumulative effect of cationic charge and folic acid present on the surface of these liposomes. In case of Dox-NTLs, cellular uptake was much lower compared to other liposomes and it could be attributed to anionic charge (approximately −20.0 mV) of the surface of Dox-NTLs. The electrostatic repulsion created by the anionic charges of liposomal surface and cellular membrane can inhibit the cellular uptake of liposome by endocytosis (Chandaroy et al., 2002). Whereas Dox-FALs showed higher cellular uptake in KB cells despite of thier anionic surface charges, which furhter confirms the role of folic acid in mediation of receptor mediated endocytosis of liposomes. These data showed that drug delivered through FA-MTLs could effectively target the KB cells. Therefore, it is regarded that presence of cationic charges along with targeting ligand on the liposomal surface provides a synergistic effect in terms of increased liposome-mediated transfection of the loaded drug.

3.6. Subcellular-trafficing of liposomes

Intracellular trafficing of the DOX loaded FALs, MTLs and FA-MTLs was investigated using confocal microscopy. Liposomes loaded with DOX were observed under confocal microscope after 2 h of incubation with KB cells. Fig. 9 shows representative composite confocal fluorescence micrographs that represent the comparative cellular uptake and distribution pattern of DOX mediated by different liposomal formulations. As shown in flow cytometry spectra presented in Fig. 8, Dox-MTLs showed relatively lower cellular uptake efficicncy in comparison to folic acid appended Dox-FALs and Dox-FA-MTLs. This response is further confirmed by fluoresence micrographs presenting significantly lower intracellular red fluoresence of DOX in case of KB cells treated with Dox-MTLs than for the cells treated with folic acid conjugated liposomes. To further elucidate the intracellular targeting potential of liposomes, a co-staining experiment was conducted by staining mitochondria green with Mitofluor and nucleus stained blue with Hoechst33342. The yellow fluorescence indicated in the confocal micrograph represents the co-localization of DOX with Mitofluor and pink fluorescence represents the co-localization of DOX with Hoechst 33342 in treated KB cells. As may be indicated that no pink fluorescence was observed inside nucleus region for cells treated with Dox-MTLs and Dox-FA-MTLs, whereas Dox-FALs imparted punctaute pink fluorescence distributed througout the nucleus of cell. Further, the co-localization fluorescence micrographs of Dox-MTLs and Dox-FA-MTLs indicates yellow fluoresence representing the co-localization of DOX and Mitofluor in treated KB cells. This indicates the specific cytosolic delivery of the STPP appended liposomes to the mitochondria of KB cells. The intracellular trafficing to mitochondria governed by miochondriopic ligand such as STPP has been reported (Weissig et al., 2006) to be due to the generation of delocalized positive charge at the surface of liposomes. This delocalized positive charge at the liposomal bilayer surface not only permits liposomes to escape endosome but also conveys the vesicle to the mitochondria in response to mitochondrial memberane potential (Ross et al., 2005). Higher fluoresence of DOX was observed with the Dox-FA-MTLs in comparison to Dox-MTLs, this further indicates the synergistic effect provided by the liposomal incoporation of dual ligands. The folic acid presented on the surface of cationically charged FA-MTLs enhanced the cellular uptake of these liposomes following receptor mediated endocytosis, as confirmed by flow cytometry. Whereas, the STPP present on the surface of these liposomes guided its endosomal escape and further controlled its trafficing to the mitochondria of KB cells.

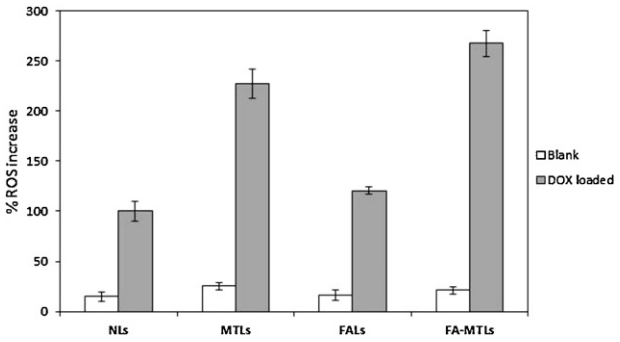

Fig. 10. Effect of liposomes on generation of reactive oxygen species (ROS) in KB cells after 24 h of incubation. Error bars represent mean ± standard deviation from three separate experiments ($P < 0.05$).

3.7. Evaluation of ROS production

Generation of ROS was measured after exposure of KB cells with Dox-NTLs, Dox-FALs, Dox-MTLs and Dox-FA-MTLs at concentration of 20 μg/ml. The results were expressed as percentage increase in fluorescence of fluorescent DCF as an index of increase in ROS generation and are presented in Fig. 10. In comparison to Dox-FALs, the Dox-MTLs showed a significant increase in ROS level, irrespective of their inferior cellular uptake in comparison to Dox-FALs. This increase in ROS generation indirectly indicates the Dox-MTLs improved accumulation of Dox in the cancer cell mitochondria. However, maximum increase in ROS levels for the cells treated with Dox-FA-MTLs was observed in comparison to all liposomal formulations. The increase ROS levels reflect the cumulative response achieved by the presence of dual targeting ligands on the bilayer surface of FA-MTLs. The folic acid present on the surface of the liposomes enhanced the intra cellular delivery of DOX, whereas STPP further regulated the mitochondrial delivery of the intracellular delivered cargo. After reaching mitochondria DOX gets activated by mitochondrial enzymes (e.g. NADH dehydrogenase) to the corresponding emiquinone that undergoes redox-cycling in air to form superoxide ($O_2^{-\bullet}$) and H_2O_2 (Davies and Doroshow, 1986). The controls used in the study (NTLs, FALs, MTLs and FA-MTLs) showed negligible effect on ROS generation indicating the ROS generation observed with formulations was indeed because of doxorubicin and further confirms the targeting potential of the STPP appended carrier system toward mitochondria of cancer cell. The result justifies the improved accumulation of DOX in mitochondria of cancer cell by indicating the increased generation of ROS (Vasquez-Vivar et al., 1997).

3.8. Evaluaion of cytotoxic activity

The comparative cytotoxicity studies between DOX loaded NTLs, FALs, MTLs and FA-MTLs liposomal formulations, were done using MTT assay. Percentage cell viability produced by different liposomal formulations at different concentrations (0.05–20 μg/ml) along with their subsequent controls are presented in Fig. 11. The IC_{50} value for Dox-FALs was found to be remarkably lower in comparison to the conventional Dox-NTLs, which were even not able to kill 50% cells even at the concentration of 20 μg/ml. A remarkable increase in the cytotoxic activity was observed with Dox-MTLs in comparison to the Dox-FALs. However, the Dox-FA-MTLs produced a significant increase in the cytotoxicity in comparison to other non-targeted and targeted liposomal formulations. The above observation justifies the finding that the targeting of DOX to the mitochondria of cancer cell using liposomes decorated with STPP as mitochondriotropic ligand and folic acid as cancer cell targeting ligand has not only modified the cellular uptake of the liposomes

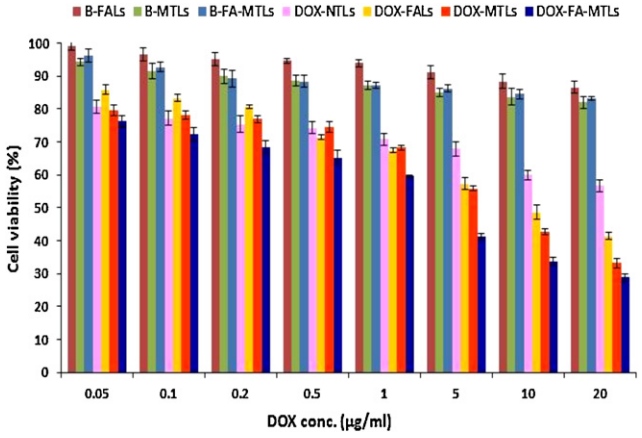

Fig. 11. Percent viability measured by MTT assay on KB cells after treatment with different liposomal formulations (0.05–20 μg/mL) for 4 h with further washing and incubation for 24 h. The data are expressed as mean ± standard deviation (SD) of three independent experiments. An OD value of control cells (unexposed cells) was taken as 100% viability (0% cytotoxicity). Statistically significant difference was observed when compared to control ($P \leq 0.05$).

but also regulated the intracellular trafficking of the carrier system, resulting in the achievement of better therapeutic effect with further reduction in dose of DOX. Empty liposomes at the same lipid concentration corresponding to that of DOX-containing liposomes at IC_{50} showed no significant toxicity.

4. Conclusion

The potential of DOX as redox cycler, for the selective killing of cancer cells through the generation of ROS was demonstrated in this study. In previous studies, folate receptor targeted liposomes (Xiang et al., 2008) and cationic liposomes (Wu et al., 2007) mediated DOX delivery have been shown to effectively target FR-expressing tumor cells. Both folic acid conjugated liposomes and cationic liposomes were able to increase the intracellular uptake of liposomes but were not able to guide the mitochondria specific delivery of DOX. In this study, mitocancerotropic liposomal carrier system was developed by achieving phospholipid bilayer incorporation of both cancerotropic ligand (folic acid) and mitochondriotropic ligand (STPP). The folic acid was incorporated to the liposomal bilayer in the form of FA–PEG–Chol conjugate, which was synthesized and characterized by thin layer chromatography and ^{1}H NMR spectroscopy. The ^{31}P NMR spectroscopy study confirmed the phospholipid bilayer incorporation of STPP. The folic acid, PEG and STPP appended mitocancerotropic liposomes present a prototype of sterically stabilized cancer cell plus mitochondria-targeted nanocarrier delivery system. The potential of which for the selective delivery of DOX to mitochondria in living mammalian cells has been demonstrated. The flow cytometry and confocal microscopy results not only revealed the potential of mitocancerotropic liposomes in increasing intracellular delivery of DOX but also confirmed DOX selective delivery to the mitochondria of cancer cell. It was further shown that DOX containing FA-MTLs had much greater cytotoxicity compared to non-targeted and targeted liposomal preparations. This indicates the synergistic effect produced by the liposomal incorporation of folic acid and STPP, which provided a cancer cell specific mitochondrial delivery of DOX.

The comparative in vitro experiments led us to conclude that optimized DOX-mitocancerotropic liposomes could be evaluated for subsequent in vivo evaluations. The novel therapeutic approach involving delivery of ROS-generating agents (DOX) showed selective toxicity in tumor cells by increasing endogenous ROS levels and raising oxidative stress over the threshold of toxicity where antioxidant systems become overwhelmed. The merger of ROS mediated anticancer therapeutics and cancer cell plus mitochondria specific nanotool warrant us to move forward to access its potential in different resistant cancer. In prospect, formulating experimental and clinically approved anticancer drugs into this dual targeted liposomal drug carrier systems can be explored to significantly improve cytotoxic and pro-apoptotic activity of drug.

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