

ORIGINAL ARTICLE

Doxorubicin loaded liposomes for lung tumor targeting: in vivo cell line study in human lung cancer

Yuehui Guo¹, Qingyun Zhu¹, Jing Shi², Yanxiang Li¹, Daiquan Fu¹, Delin Qiao¹, Shiwei Chen¹, Yuwei Yang¹, Yane Wang¹

¹Department of Intervention, Shanghai Pudong New Area Gongli Hospital, Shanghai, 200135, China. ²Department of Gastroenterology, Shanghai University of Medicine and Health Sciences Affiliated Zhoupu Hospital, Shanghai, 201318, China.

Summary

Purpose: In the present investigation the hydrophilic drug doxorubicin (DOX) was successfully incorporated with the drug carrier GE11 which serves as marker for tumor cells in non small cell lung cancer(NSCLC) to form the liposomal formulation.

Methods: The formulation was fabricated in two steps: one being the preparation of liposomal formulation using reverse phase evaporation method and second is synthesis of DSPE-PEG₂₀₀₀ - GE 11 complex.

Results: Thus prepared liposomes when evaluated via scanning electron spectroscopy showed smooth and spherical surface with particle size ranging between 102±0.3 to 120±0.5 nm. The percent encapsulation efficiency was 65.34 with highest drug release of 98% up to 45 h. The cytotoxic study

revealed the non-toxic nature of carrier protein (i.e. GE11). The microbiological study has shown the antibiotic efficiency of liposomal formulation to be comparable with pure drug. In vivo cellular uptake study showed efficiency of GE11 protein in accumulation in tumor cells. The study conducted in mice showed more reduction in tumor size with liposomal formulation (312 mm³) than with pure drug (540 mm³).

Conclusion: DOX loaded liposomes with GE11 as carriers were successfully formulated by using reverse phase evaporation method. The prepared liposomal formulation was found to be most effective in combating cancer cells when compared to pure drug.

Key words: doxorubicin, GE11 protein, liposome, lung cancer

Introduction

Lung cancer is the leading cause of cancer-related death among men worldwide and the second among women [1], with a 5-year survival rate of only 18%. About 13% of lung cancers are small cell lung cancers (SCLC) while non-small cell lung cancer (NSCLC) accounts for 87% of the cases. In NSCLC, primary treatment comprises surgical resection. Most of the cancers are diagnosed at an advanced stage for which platinum-based chemotherapy could be the only option [2]. Only 30% of patients respond positively to this kind of chemotherapy, side effects being a severe cause in therapy

failure. Targeting of the drug to specific cell receptors can be best available option to combat the side effects. Tyrosine kinase inhibitors (TKIs) allow EGFR mutation in patients and serve as one of the important diagnostic and novel therapeutic targets when needed. The prime reason for lung cancer genesis is long-term tobacco chewing/smoking [3,4]. For non-smokers the reason for lung cancer genesis can be a genetic factor or exposure to toxic gases, asbestos and/or other forms of air pollutants. Passive smoking is one reason for developing lung cancer in non-smokers which occurs when healthy

Corresponding author: Shiwei Chen, MD. Department of Intervention, Shanghai Pudong New Area Gongli Hospital, Rd no.219, Miaopu, Shanghai, 200135, China.
Tel/Fax: +86 021-58858730-5197, Email: charlenasiente@yahoo.com
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people continuously come in contact with smoke. Computed tomography (CT) scan or X-rays of chest can be diagnostic means. Biopsy is considered the final confirmatory test performed by bronchoscopy or CT-guidance. Some of the common treatments for lung cancer include surgery, chemotherapy, and radiotherapy. Most of the times NSCLC is treated with chemotherapy whereas SCLC is controlled via chemotherapy and radiotherapy [5,6]. The line of treatment depends on the type of cancer and the stage or degree of disease spread. The patient overall health matters also. The preventive measures include avoidance of risk factors like smoking and air pollution. Accumulated past experience proves that lung cancer is a non-curable disease [7].

Doxorubicin hydrochloride (DOX), also known as Adriamycin, is used to treat various types of cancers like bladder cancer, breast cancer, gastric cancer, Kaposi's sarcoma, lymphoma, stomach ulcers, soft tissues and osteogenic sarcomas etc. Sometimes it can be used in the treatment of Ewing's tumor, squamous cell carcinoma etc. DOX belongs to anthracycline antibiotics, natural products synthesized from the soil fungus *Streptomyces* [8]. The anti-cancer activity of DOX involves interaction with DNA and macromolecular biosynthesis which is responsible for inhibition of topoisomerase II that is responsible for DNA supercoils in transcription. This prevents DNA double helix from resealing, thereby stopping the replication process. The drug can be administered bolus intravenously while sometimes infusion is preferable albeit taking longer time [9].

The liposomes are spherical vesicles having at least one lipid bilayer for administration of nutrients and pharmaceutical drugs. Phospholipids, especially phosphatidylcholines, are main component of liposomes [10]. Other lipids such as egg phosphatidyl ethanolamine can be formulated if compatibility is no issue with lipid bilayer. Most of the times liposomes have large multilamellar vesicles (LUV) and cochleate pocket. Liposomes

are biocompatible, biodegradable and non-toxic material by their composition and thus are potential and useful for targeted delivery of drugs [11]. The size of the liposomes ranges from 10-20 nm engulf with aqueous and/or multiple lipid bilayer. The liposomes are capable of engulfing large varieties of molecules such as drugs, radiolabelled nuclei, genes etc. Significant breakthrough has been achieved in liposomal formulations for drug delivery applications and few liposomal drugs, that are antifungal and antitumor, have been approved for clinical use [12,13].

Tissue necrosis or tissue toxicity is a major problem of chemotherapeutic agents. Targeting of drug to an infected site or tumor tissue is the only way and major challenge to avoid toxicity. Some of the studies show that cancer cells leak from their surface various proteins like HSP47 or CBP2 which are present in ER Golgi complex of tumor cells and serve as markers for the drug to target the tumor site [13].

In the present study the DOX has been engulfed in liposomal formulation in order to target tumor cells in lung cancer patients.

Methods

DOX was kindly procured from Shouguang Fukang Pharmacy Factory (Shandong, China). Egg phosphatidylcholine (EPC) and dipalmitoyl phosphatidylcholine were kindly procured by Lipoid GmbH, Ludwigshafen Germany. Disodium hydrogen phosphate, potassium dihydrogen phosphate, DSPE-PEG₂₀₀₀- Maleimide, sucrose and chloroform were purchased from Sigma Aldrich, USA. All other materials were of analytical or reagents grade.

Statistical design

2X3 full factorial statistical design was used for the development and optimization of the DOX liposomes. EPC (A), Cholesterol (B) and Chloroform (C) were selected as independent variables and EE (A) and percent (DR) were selected as dependent variables. Second order

Table 1. Formulation data and evaluation parameters of DOX-LP/GE11

Batch No.	EPP(gm)	Cholesterol (gm)	DOX solution (ml)	% Encapsulation efficiency	% Drugs release	Particle size (nm)
D1	0.25	0.10	0.10	63.46	93.99	102 ± 0.3
D2	0.25	0.50	0.20	64.23	96.12	112±0.2
D3	0.75	0.10	0.10	64.36	96.86	118±1.2
D4	0.25	0.10	0.20	63.38	95.98	119±0.9
D5	0.75	0.50	0.10	64.87	97.34	115±0.5
D6	0.75	0.10	0.20	64.54	96.67	118±0.7
D7	0.25	0.50	0.10	63.92	95.48	120±0.5
D8	0.75	0.50	0.20	65.34	98.56	116±0.3

polynomial equation and contour plots were generated to predict the responses. Different levels of dependent variables are shown in Table 1. Design Expert software version 6.0.8 was used. The formulation bathes are shown in Table 2.

Synthesis of DSPE-PEG₂₀₀₀ - GE11 complex

The method for synthesis for DSPE-PEG₂₀₀₀-GE11 complex is a slight modification of previous process reported by Cheng et al. Pre-weighed quantity of DSPE-PEG₂₀₀₀- Maleimide (Mal) (10 mg, 0.0075 mmol) was dissolved in distilled water with subsequent addition of GE11 (9 mg). The system was kept at room temperature to allow completion of reaction at least for 24 h. Gentle stirring was done and end point was monitored using thin layer chromatography (TLC). The disappearance of spot of DSPE-PEG₂₀₀₀- Mal was determined as end point of reaction.

Preparation of liposomal formulation of DOX

Reverse phase evaporation was used for preparation of liposomes. The required amount of DOX was dissolved in chloroform solution. Pre-weighed amount of EPC and cholesterol were dissolved in chloroform (in which the drug was dissolved previously) in round bottom flask (solution A). DOX solution was added slowly into the solution A up to 5 min sonication at room temperature. Rotary evaporator was used under reduced pressure to remove organic solvent with subsequent addition of water for hydration. The resulting emulsion was kept stand for complete hydration and after 24 h drug-loaded liposomes were separated by centrifugation. The thus formed liposomal emulsion was dried and prepared liposomes were collected [14].

Preparation of DOX loaded GE11 liposomes (DOX-LP/GE11)

The DOX-loaded GE11 liposomes (DOX-LP/GE11) were prepared using two step method. The first step involves formation of DOX liposomes using reverse phase evaporation (RPE) method. In this method first W/O emulsion is formed from two phases as EPC and chloroform (in which the drug is previously dissolved) by ultra-sonication process. The residual solvent is removed under reduced pressure to form liposomes. Dua et al have reported the method to be with highest encapsulation efficiency amongst other methods of preparation of liposomes. The method is reported to be best suitable for encapsulating small and large macromolecules like proteins etc. In the second step the liposomes are mixed with the solutions of different molar ratios of DSPE PEG-GE11 solution. The system is incubated at 56°C yielding DOX-LP/GE11.

Evaluation of doxorubicin loaded liposomes [15-17]

Morphology of liposomes

Scanning electron microscope was used to observe the shape, size, and surface morphology of liposomes. Small quantity of liposomal suspension was mounted on brass stub and made electrically conductive.

Drug loading

About 5 ml of liposomal suspension was used to analyze free DOX which was separated using Sephadex G-50 mini column. The suspension was centrifuged at 5000 rpm for 15 min. A 0.1% v/v solution of Triton X-100 was used to digest the elute obtained. The solution obtained was analyzed by UV spectrophotometer at 240 nm.

Particle size determination

Horibunano particle analyzer was used to determine the particle size of liposomes using dynamic light scattering technique (also known as photon correlation spectroscopy). The method is precise in determining particles up to 1 nm. A droplet of liposome formulation was mounted on glass slide and placed on mechanical stage of microscope, then the globule diameter was measured and recorded for 100 globules and the average particle size was determined.

Encapsulation efficiency in liposomes

About 100mg of liposomal powder were taken and added in absolute alcohol to carry out the lysis of liposomes. This dispersion was further sonicated for 10 min to complete removal of the drug from the liposomes and then filtered through membrane filter (0.45µm) and estimated for drug content by UV spectrophotometer at 240 nm. The drug entrapment efficiency was calculated using the formula:

$$\text{Drug Encapsulation Efficiency} = \frac{\text{Estimated \% drug content}}{\text{Theoretical \% drug content}} \times 100$$

In vitro diffusion study

Modified Franz diffusion apparatus was used to study *in vitro* diffusion of DOX. The receptor compartment of 20 ml capacity with dialysis membrane previously soaked overnight in phosphate buffer pH 7.4 (molecular weight cut off 12000 to 14000 Da from Hi-Media Laboratories Pvt. Ltd, having flat width of 24.26 mm and diameter of 14.3 mm with approximate capacity of 1.61 mL/cm) attached to one end used for study. The dialysis membrane was mounted in between donor and receptor

Table 2. Microbiological data of DOX-LP/GE11 and standard solutions of pure drug

Formulation	Diameter of zone of inhibition (mm)	% Inhibition
Standard solution 1 (1 µg/ml)	15	71.43
Standard solution 1 (3 µg/ml)	21	100.00
DOX-LP/GE11	18	85.72

cell. Receptor cell was filled with phosphate buffered saline (PBS) pH 7.4 such that it touches the membrane surface. The setup was assembled on magnetic stirrer with rotation speed of 50 rpm and temperature $37 \pm 0.5^\circ\text{C}$. The aliquots of 2 ml were withdrawn at different time intervals and replaced with fresh PBS, pH 7.4, each time. The samples were analyzed using UV spectrophotometer at 240 nm.

Microbiological assay

Cup plate method was used to carry out microbiological assay of DOX in which potato agar medium was inoculated with *C. Albicans* micro-organisms and poured in Petri plates for solidification. Wells of about 10 mm diameter were made on solidified agar gel by using cy-

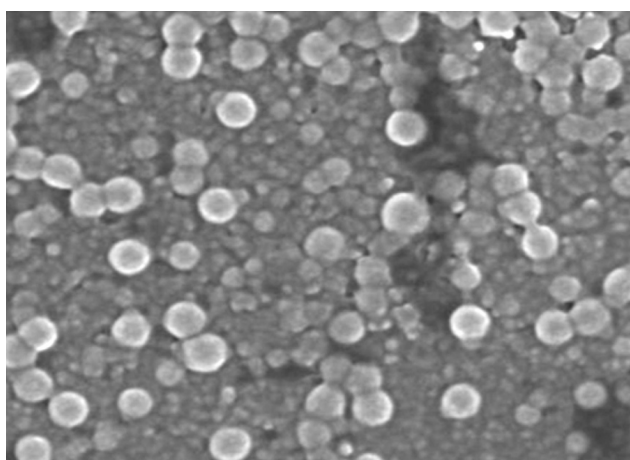


Figure 1. Surface morphology of DOX-LP/GE 11 through scanning electron microscopy.

lindrical wall borer and the sample solution (500 μL) was poured in wells. To compare standard solutions, DOX was prepared at a concentration of 1 $\mu\text{g}/\text{ml}$ and 3 $\mu\text{g}/\text{ml}$. The zone of inhibition was observed after 48 h and the diameter was measured and recorded.

Cytotoxicity test

The MTT assay method was employed to study the cytotoxic profile of DOX liposomes. H 1299 cells were cultured using 10% (v/v) fetal bovine serum (FBS) at 37°C with 5% CO_2 in humidified incubator. The cells were cultured in the form of monolayer, and FBS was changed every alternate day. Such cultured cells were seeded in 96-well plates with density of 8×10^5 cells/well up to 24 h. After a day, FBS was replaced with DOX-liposomal solution (5 $\mu\text{g}/\text{mL}$). Then, after 48 h MTT solution (100 μL as 0.5 mg/mL) was added to each well and allowed for incubation for 4 h. Alas Dimethyl Sulfoxide (100 μL) was added to each well and the absorbance of each plate was calculated using microplate reader. The control samples were used for calculating the cell viability.

Cellular uptake study

Confocal microscopy method was used to study the cellular uptake of DOX by H 1299 cells. Firstly, 1×10^4 H1299 cells were seeded in 24-well plates and cultured for 2 days. These cells were again incubated with DOX liposomal solution (5 $\mu\text{g}/\text{mL}$ diluted in serum free medium) for 2-4 hrs. Then, FBS was replaced with Hoechst 33342 for 25 min. In the last stage, PBS (pH 7.4) was used to rinse the cells three times and observed under confocal microscope at 40x magnification at 488 nm using UV spectrophotometer.

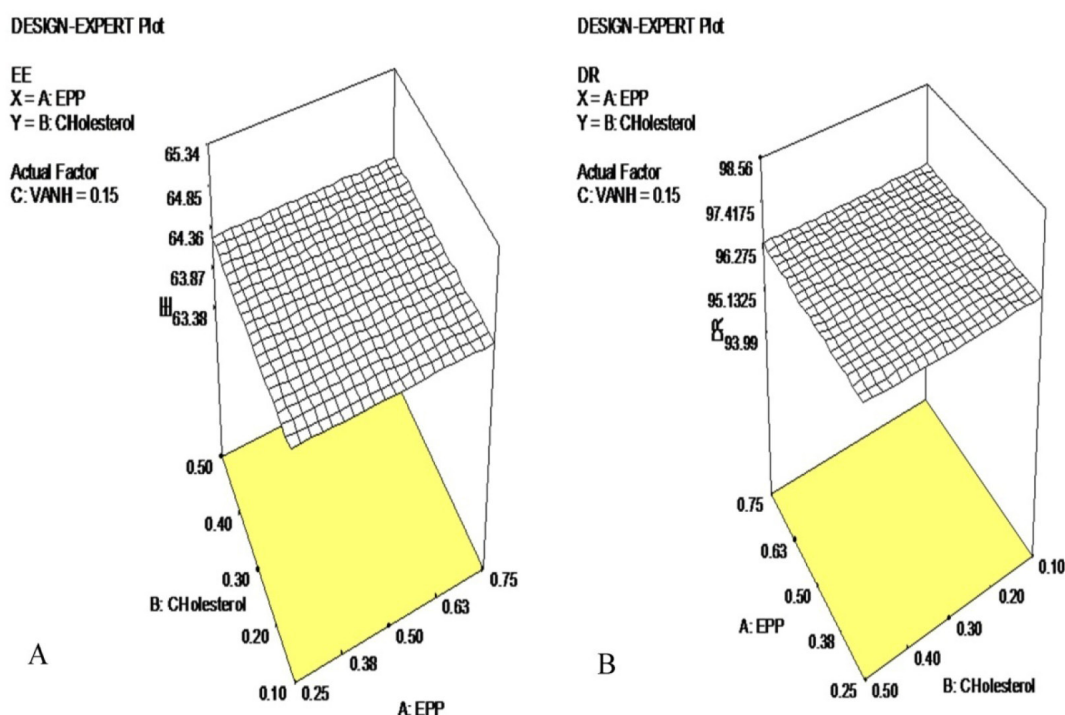


Figure 2. Counter plots showing the effect of independent variables on A) % encapsulation efficiency, and B) drug release.

Intracellular distribution of liposomes

Confocal laser scanning microscopy was used to study the intracellular distribution of DOX liposomes. A glass cover slip was used to seed the H 1299 cells and placed on 6-well plates. These were cultured for 24 h at room temperature. On completion of 24 h the cells were added with a solution of DOX liposomes and incubated for 1-2 h. Lyso tracker green solution was added for an hour and Hoechst 33342 for 25 min after removal of the medium. Lastly, the cells were treated with 5% formaldehyde solution for 20 min to fix cells and observed under confocal laser microscope.

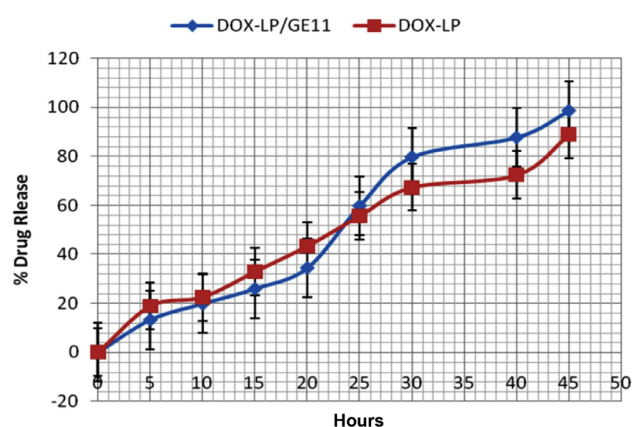


Figure 3. In vitro diffusion study of DOX-LP/GE 11 in phosphate buffer saline solution (pH 7.4) ($p < 0.05$).

Study on nude mice

The nude mouse model was selected for the study. The mice were injected with injections of KLN-205 cells, 4 days prior to injections of tumor were given single i.v. injections of 5 mg/kg of Pure DOX, DOX-LP/GE11. After 40th day of treatment. Animals were sacrificed and sample of lung tissue was taken from these two treatment groups. The tumor lobes were identified and dissected, fixed in 10 % buffered formalin solution of paraffin blocks. The sections of approximately 5 μ m were cut off and stained with hematoxylin and eosin.

Statistics

All statistical evaluations were performed using Design-Expert[®] software, version 6.0.8. The statistical methods used like Counter Plot were derived using the same software only.

Results

The formulation and evaluations of physical properties of these liposomes are shown in Table 1. The surface morphological studies using scanning electron microscopy are shown in Figure 1. The surface of DOX-LP/GE11 was found to be spherical with smoother surface. These uniform surface characteristics of liposomes may often result in increased encapsulation efficiency of DOX. The particle size of the DOX-LP/GE11 ranged from

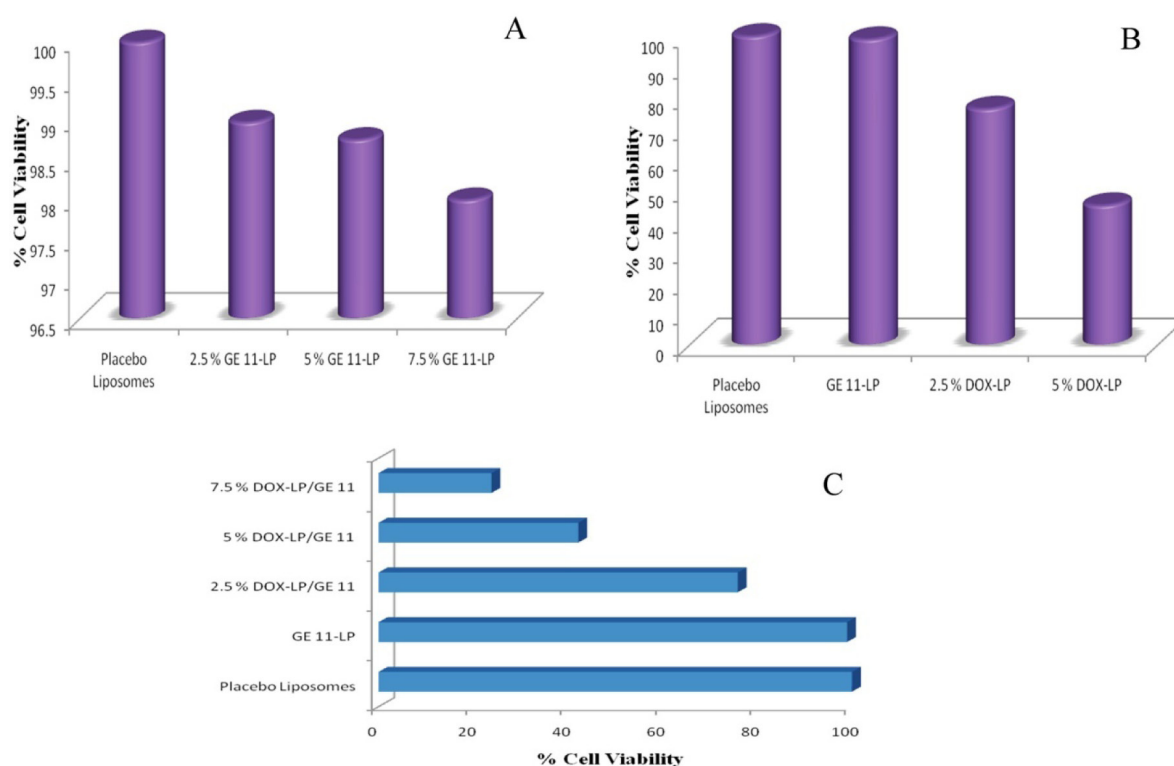


Figure 4. Cytotoxic study of DOX-LP/GE 11. **A:** GE11 had not significant cytotoxic effect of various concentrations on cancer cells. **B:** The pure DOX LP shows more cytotoxicity when compared to carriers and placebo. **C:** With increasing concentration of DOX the cytotoxic effect goes on increasing.

of 102 nm to 120 nm. The PDI was 0.412, which is closer to zero indicating monodispersed and narrow size distribution of liposomes. The particle size of the DOX loaded liposomes ranged from 150.5 nm to 180.35 nm with average particle size of 165.80 nm. The PDI value of 0.315 was very close to zero which indicates the monodispersed and narrow size distribution of the liposomes. The percent encapsulation efficiency of DOX-LP/GE11 ranged from 63.84 to 65.34. The amount of percent drug encapsulated was determined from the amount of free drug present in supernatant after centrifugation of liposomal suspension. From the values present in Table 1 it can be predicted that the percent EE varied with change in the concentration of the independent variables. The counter plot showing effect of independent variables on percent EE can be seen in Figure 2. *In vitro* diffusion study of liposomes was done in PBS (pH 7.4). The formulation showed initial release about 14.52% which is considered as initial burst release. This may be due to presence of outer layer of liposomes which dissolves rapidly when comes in contact with the solvent. This burst release is followed by sustained release of drug up to 86.48% at the end of 45 h as indicated in Figure 3.

The microbiological assay was done in order to compare the efficacy of DOX-LP/GE11 with standard solution of pure drug. The zones of inhibition of liposomal formulation were compared with these standard solutions as shown in Table 2. The cytotoxic study results of MTT assay are shown in Figure 4. To study the effect of carrier on cancer cells, the cytotoxicity of GE11 liposomes were determined first and the results obtained are shown in Figure 4A. The results clearly show that GE11 protein has no significant cytotoxic effect on cancer cells. All the formulations of GE11 had high cell viability (almost greater than 97%) which is indicative of the non-toxic nature for liposomal carrier and GE11. GE11 liposome showed more cytotoxicity than placebo liposomes. The cytotoxicity of pure drug DOX was compared with carrier and placebo liposomes which proved the cytotoxic nature of DOX (Figure 4B). The pure DOX liposome showed more cytotoxicity when compared to carriers and placebo. The cytotoxic effect of different concentration of DOX-LP/GE11 showed that with increasing concentration of DOX the cytotoxic effect goes on increasing (Figure 4C). Cellular uptake of the liposomes was evaluated using confocal microscopy and the results are summarized in Figure 5. The H1299 cells treated with DOX-Lp/GE11 showed intensified fluorescence which increased after 1 h and 2 h, which shows that GE11 is highly capable of cellular uptake of the liposome. It has

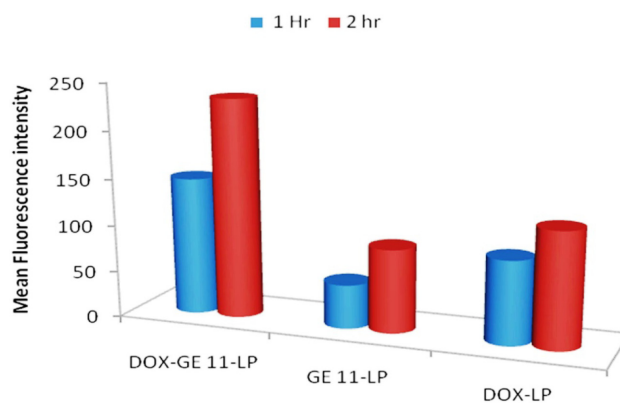


Figure 5. Flow cytometric evaluation of liposomes for cellular uptake of DOX-LP/GE11 by H1299 cells. Liposomes with GE11 part of composition show highest cellular uptake ($p < 0.05$).

also been noted that the intensity of fluorescence becomes stronger with the prolongation of culture incubation time indicating time dependent cellular uptake of liposomes. It was found that the nodule numbers were almost the same in healthy mice and in mice treated with DOX-LP/GE11. The numbers of nodules in mice injected with pure DOX were slightly more in number than healthy subjects and DOX-LP/GE11 injected mice. The *in vivo* anti-cancer efficacy of DOX, DOX-LP/GE11 was studied in H1299 cells bearing group of mice. Tumor cells in the control group showed tumor volume 842 mm³, while in those with pure drug solution the tumor volume was only 540 mm³ and in the group administered liposomal formulation it was 312 mm³. When tumor volume is considered, the liposomal formulation was almost 3 times more efficient when compared to the control group and the pure drug formulation.

Discussion

Owing to variable particle size and biocompatible lipid membrane liposomes are the choicest ones amongst targeted drug delivery system and efficient in delivering large variety of drug molecules along with protein, biomarkers, and radiolabelled nuclei etc. Due to numerous features of liposomes like accumulation in targeted tissue, the drug molecule directly gets localized in tumor cells of lung which could result in enhanced sensitivity to chemotherapy. The major disadvantage of conventional system of causing tissue necrosis on non-cancer cells due to direct exposure of tissue surface to drug can be overcome by using liposomes as the drug molecule is embedded in the lipidic wall. Releasing drug in sustained manner so as to prolong the drug release at targeted site is one of the

best known advantages of liposomes [19]. In many solid tumors, cell growth mediated by receptors is responsible for numerous signal transductions which indeed help targeting these receptors with anticancer drugs and thus help in chemotherapy. Colloidal drug delivery system proves to be one of the best methods for such targeted delivery. The EGFR is targeted using GE11 peptide. GE11 with molecular weight 72.63 g/mol is a dodecapeptide which shows terrific affinity to EGFR. The literature studies have shown that EGFR levels on tumor cell surface do not deplete even after treatment with GE11 polyplexes, which confirms the fact of EGFR recycling with longer receptivity of cells circulating GE11 polyplexes. GE11 protein was conjugated to DSPE-PEG₂₀₀₀-Mal using addition reaction. The degree of conjugation was determined using HPLC. Appearance of additional peak confirms the formation of dimer of GE11 due to the presence of thiol group.

The drug is released from liposomes in sustained manner which is important for prolonging the therapy in cancer patients. Cancer treatments usually take long time, therefore sustained release of drug is a very much crucial factor in tumor targeting. The release of drug usually follows a distinct pattern that can be quantified with mathematical models like zero order, first order, Higuchi and Koremeyer-Peppas model. For the present release

pattern the Higuchi model was found to be best fit, which indicated the diffusion type of release of drug from dosage form. The drug release follows diffusion and erosion type of pattern of release.

Conclusion

DOX loaded liposomes with GE11 as carriers were successfully formulated by using the reverse phase evaporation method. The size and surface morphology of liposomes was found to be suitable for targeted drug delivery. The polymers used in the formulation process were good enough to show highest encapsulation efficiency and releasing drug in sustained manner for prolong period of time (45 h). The prepared liposomal formulation was found to be most effective in combating cancer cells when compared to pure drug.

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Conflict of interests

The authors declare no conflict of interests.

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