ORIGINAL ARTICLE

Surface Modified Solid Lipid Nanoparticles for Brain Cancer Treatment

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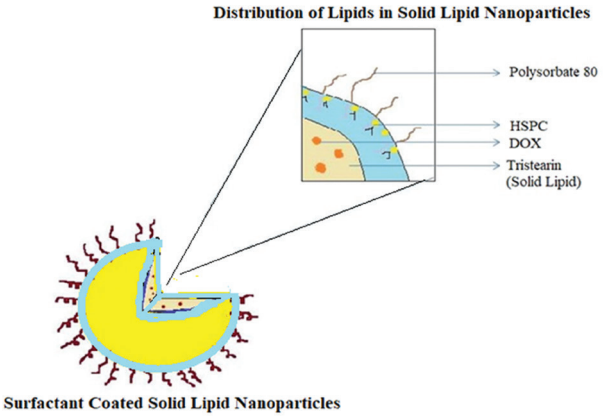
Abstract

Introduction: Primary brain tumors are the tumors that grow within the brain from the brain cells. The treatment of primary brain tumor is the biggest challenge in the field of oncology due to the presence of blood–brain barrier (BBB). The BBB prevents the entry of most of the drugs inside brain cells from the blood. Therefore, the present work was intended to develop and investigate the ability of polysorbate 80 coated solid lipid nanoparticles (SLN) to deliver doxorubicin (DOX) in the brain for the treatment of brain cancer cells. **Materials and Methods:** The polysorbate 80 coated, DOX loaded-SLN (DOX-SLN) were prepared by the solvent evaporation method using a mixture of tristearin, hydrogenated soy phosphatidylcholine, and cholesterol lipids. DOX-SLN were characterized for various attributes such as average particle size, size distribution, zeta potential, surface morphology, entrapment efficiency, drug release, cytotoxicity, and cellular uptake studies. Cytotoxicity and cellular uptake studies were performed on U87MG brain cancer cell lines. **Results and Discussion:** DOX-SLN prepared by solvent evaporation method possesses an average particle size near 200 nm and polydispersity index (PDI) below 0.3. The zeta potential of the prepared nanoparticles was found to be -14 mV with 45.3 ± 0.2% entrapment efficiency. The cytotoxicity studies showed the higher toxicity of DOX-SLN than plain DOX on U87MG cell lines. The cellular uptake studies also confirmed the internalization of the DOX-SLN inside brain cancer cells. **Conclusions:** The results confirmed the development of a potential carrier for brain cancer treatment with enhanced ability to cross the BBB.

Key words: Brain cancer, doxorubicin, polysorbate 80, solid lipid nanoparticles, tristearin

Graphical abstract



Representation of polysorbate 80 coated solid lipid nanoparticles

INTRODUCTION

Primary brain tumors are the tumors that grow within the brain from the brain cells. The treatment of primary brain tumor is the biggest challenge in the field of oncology due to the presence of blood–brain barrier (BBB). BBB is a tight endothelial membrane which protects the brain against toxins, viruses, chemical compounds, etc. This behavior of the

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BBB creates a problem during the brain cancer chemotherapy as >98 % drugs will not cross BBB.[1] The standard treatment for brain cancer includes maximal surgical rejection followed by radiotherapy and chemotherapy. However, despite continued research and new approaches, the prognosis for patients with malignant brain tumors is still extremely poor.[2] Hence, there is need of extensive research for the treatment of brain cancer. Therefore, with reference to brain cancer therapy, it becomes very crucial to develop nano-particulate systems which can cross the BBB.

In the proposed work, it was aimed to develop the polysorbate 80 coated doxorubicin-solid lipid nanoparticles (DOX-SLN) loaded with DOX for drug delivery to brain cancer cells. DOX is, a potent anticancer agent, widely used for the treatment of cancer. DOX belongs to anthracycline family having wide antitumor spectrum.[3,4] DOX inhibits cancer cells growth by intercalating with DNA and prevent the topoisomerase II-mediated DNA repair.[5,6] However, the most frequent drawback of DOX is the onset of drug resistance, which is due to its active efflux through P-glycoprotein (Pgp).[7] The clinical use of DOX is also limited due to its lethal cardiotoxicity.[8] Delivery of DOX through polysorbate 80 coated SLN can decrease the cardiac toxicity of anthracyclines by reducing their distribution in cardiac tissues as well as inhibit active efflux through Pgp.

The surfactant-coated SLN improves drug transportation through enhanced permeability due to surface activation, receptor-mediated nonspecific adsorption, and inhibition of Pgp efflux [7] SLN are the particles of nanometric sizes composed of lipids/lipid mixtures, solid at body temperature and stabilized by one or more surfactants.[9] SLN are bio acceptable, biodegradable and can be taken up readily by the brain.[10,1] SLN emerged as a potential carrier for cytotoxic drugs.[11] Supplemented with small size as well as surfactant coating prolongs the circulation time in blood, feasible scale up for large scale production and absence of burst effect makes them interesting candidates for study. The surface modification of the SLNs with polysorbate 80 augments the delivery of the nanocarrier across the BBB. Therefore, in the present work, it was hypothesized that the DOX-SLN will cross the BBB due to the presence of polysorbate 80 on the surface and delivers the drug to the cancer cells.

MATERIALS AND METHODS

Materials

DOX was purchased from HiMedia, Mumbai, India. The solid lipid tristearin and steroid type of lipid cholesterol were purchased from Sigma-Aldrich, Germany. The phospholipid hydrogenated soy phosphatidylcholine (HSPC) was provided as a gift sample from Lipoid, Germany. All other chemicals and reagents used were of analytical grade.

Preparation of SLN

SLN were prepared according to the method reported by[12] with minor modifications [Figure 1]. Briefly, SLN were prepared by solvent evaporation method at a concentration of 10 mg/ml lipids in ethanol. The lipid phase was prepared by dissolving the mixture of drug and lipids (Tristearin: HSPC: Cholesterol; 1:1.5:1.2) in ethanol at temperature 70°C. The aqueous phase was prepared by the addition of polysorbate 80, (0.5 % v/v) in 20 ml phosphate buffer saline (PBS, pH 7.4). The aqueous phase was also heated at the same temperature. The lipid phase was gradually added (25 drops/min^{1}) into aqueous phase using syringe (22-gauge needle speed) under high speed stirring (2000 rpm) over magnetic stirrer (Remi Equipments, Mumbai, India) followed by homogenization in an ultrasonication probe during 2 min at 70% amplitude (PCI, India). The SLN were concentrated by centrifugation at 20,000 rpm for 30 min and again suspended in fresh 20 ml PBS (pH 7.4) containing 1% of polysorbate 80. The suspended SLNs were again stirred for 1 h on a magnetic stirrer at ambient temperature. The unentrapped drug was removed by passing the suspension from Sephadex G-50 minicolumn.

Characterization of SLNs

Particle size, PDI, and zeta potential

The average particle size, particle size distribution PDI, and zeta potential of DOX-SLN were determined by photon correlation spectroscopy (PCS) with a Zeta Sizer (Malvern Instruments, UK), equipped with the Malvern PCS software. Previous to the analysis, the formulation was diluted in distilled water.

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The amount of entrapped drug was estimated by the method reported by Gulbake *et al*., 2010[13]. The DOX-SLNs (free from

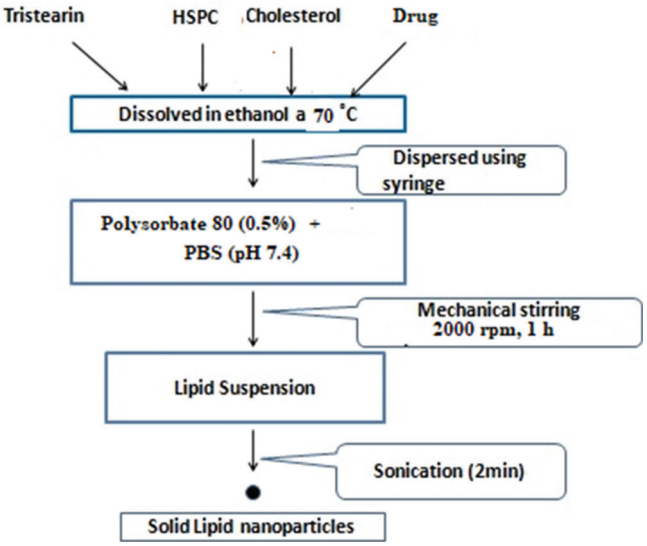

Figure 1: Schematic representation of the method of preparation of solid lipid nanoparticles

the unentrapped drug) were lysed by Triton X-100 (0.1% v/v) and filtered. The absorbance of the filtrate was taken at 480 nm using Uitavioatiolet (UV) spectrophotometer. Percent of drug entrapment was calculated using the formula shown below.

$$\text{Percent (\%) drug entrapment} = \frac{\text{Amount of drug released from the lysed SLNs}}{\text{Amount of drug initially take to prepare the SLNs}} \times 100$$

Surface morphology of SLNs

The surface morphology of the DOX-SLNs formulations was determined by scanning electron microscopy (SEM; JEOL JSM 35C) at 15 keV and transmission electron microscopy (TEM; Philips, Tecnai 20, Holland) at 200 kV acceleration voltages and 50,000X magnification. Samples were prepared by diluting the formulations with distilled water. For the TEM method, the sample was stained with 3% solution of phosphotungstic acid in water and then examined.

In vitro drug release studies

In vitro drug release from the DOX-SLNs formulations was determined by a diffusion method using a dialysis bag (MW, 3500 DA, HiMedia, Mumbai, India). The DOX-SLN suspension (containing the drug equivalent to 5 mg) free from any unentrapped drug was filled in a dialysis bag, sealed, and suspended in 50 ml PBS (pH 7.4) taken in a beaker with continuously stirring at a constant speed. The temperature was maintained at 37 ± 2°C. At regular time intervals, i.e., 1, 2, 4, 6, 8, 12, 24, and 48 h samples (1 ml) were withdrawn to determine absorbance using UV spectrophotometer at λ_{max} of 480 nm DOX-SLNs. Simultaneously, the volume was replaced with the same volume of fresh buffer solution.

Cytotoxicity and Cellular uptake studies

The cytotoxicity of DOX and DOX-SLNs against brain cancer cells was assessed by the sulforhodamine B (SRB) assay method.[14] For the cytotoxicity study, U87 MG cells were procured from the National Center for Cell Sciences, Pune, India, and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine. The cells were plated in 96 well microtiter plates and incubated for 24 h at 37°C, 5 % CO_2, 95% air, and 100% relative humidity. The cells were then incubated with the formulations in final drug concentrations, i.e. 0.1 µg/ml, 1 µg/ml, 10 µg/ml, and 100 µg/ml at standard conditions for 48 h. The cells were fixed by the addition of 50 µl of 30% (w/v) cold trichloroacetic acid and again incubated at 4°C for 1 h followed by washing with water and drying. Each well was stained with 50 µl of 0.4% (w/v) SRB solution in 1% v/v acetic acid followed by incubation for 20 min at room temperature. After staining, excess stain was removed using 1% v/v acetic acid and dried. The bound stain was removed with 10 mM trizma base, and the absorbance was recorded at a wavelength of 540 nm on microplate spectrophotometer (Model 680, Bio-Rad, Japan). The viability of cells was expressed in terms of percentage compared to untreated cells (control).

The cellular uptake study was also performed on U87MG cells using confocal microscopy. U87MG cells were cultured in Petri dishes by taking the same medium as used for the cell cytotoxicity study, until density reached up to 80% confluence. The medium was replaced with plain coumarin 6 (C6) and C6 loaded SLN (SLN C6) and incubated for 2 h. After incubation, the dispersion was removed. The cells were fixed by the addition of 1 ml of 70% v/v ethanol solution at 37°C. After 20 min, the ethanol solution was removed, and cells were washed 3 times using PBS (pH 7.4). Then, 10 µl of 5 mg/ml propidium iodide (PPI) was added to stain the nucleus. After 30 min, stain cells were again washed 3 times with PBS (pH 7.4). The coverslip is carefully removed and inverted on a clean slide to observe fluorescence. The same procedure was followed to study the uptake after 4 h. Cell-associated fluorescence was determined using fluorescence-activated cell sorters instrument. The samples and PPI stained cell nucleus were found to be in blue color and red color, respectively.

Table 1: Particle size and entrapment efficiency of the DOX-SLN

Formulations	Particle size (nm)	PDI	Zeta potential	Entrapment efficiency (%)
DOX-SLN	202.2±1.2	0.2440±0.05	−14.8±1.43 mV	45.3±0.2

DOX-SLN: Doxorubicin-solid lipid nanoparticles

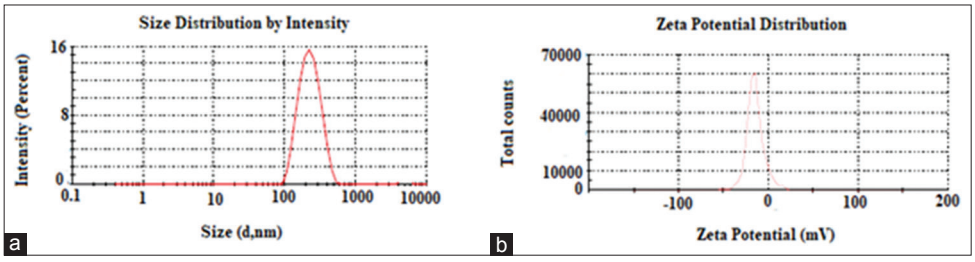

Figure 2: (a) Particle size distribution (b) zeta potential distribution

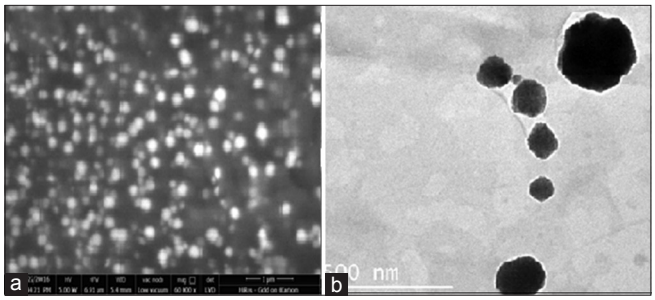

Figure 3: Photomicrographs of doxorubicin-solid lipid nanoparticles obtained by (a) scanning electron microscopy (b) transmission electron microscopy

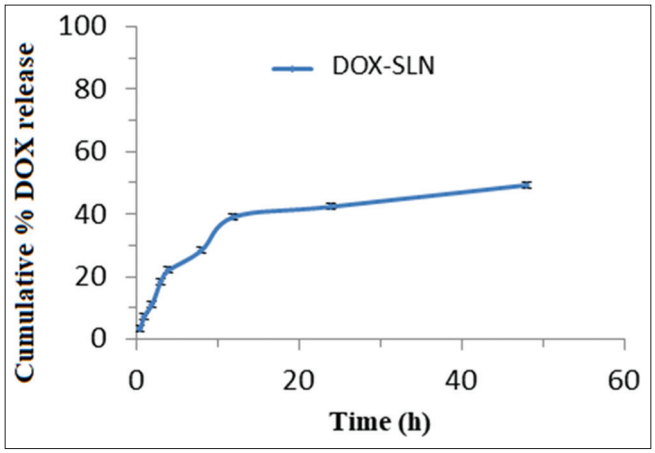

Figure 4: *In vitro* drug release of the doxorubicin-solid lipid nanoparticles ($n = 3$; $P \leq 0.05$*)

Statistical analysis

The data obtained from the experiments were analyzed by analysis of variance, lack of fit tests and multiple correlation coefficients. Student's t-test was used to test the statistical significance wherever applicable. Obtained data were expressed as mean±SD ($n = 3$).

RESULTS AND DISCUSSION

DOX-SLN were prepared by the mixture of lipids, i.e., Tristearin, HSPC, and Cholesterol by the solvent evaporation method. The heterogeneous lipids mixtures will not form crystals and provide stability to the SLNs. During SLNs formation, ethanol travels across the ethanol-lipid phase in the aqueous phase. The evaporation of ethanol at 70°C causes rigidization of the lipid phase leading to the formation of SLNs. The prepared DOX-SLN were characterized for the particle size, PDI, zeta potential, % EE, and surface morphology. The results are shown in Table 1. The average particles size of formulation DOX-SLN was found to be 202.2 ± 1.2 nm with 45.3 ± 0.2 %EE. The zeta potential of SLN was found to be -14.8 ± 1.43mV [Figure 2]. The SLN with slight negative surface charge has high ability for drug delivery to brain in comparison to positive surface charged SLN.[15] The negative surface charged nanoparticles also have high stability and less toxicity than positively charged nanoparticles.[16] The PDI value of DOX-SLN was

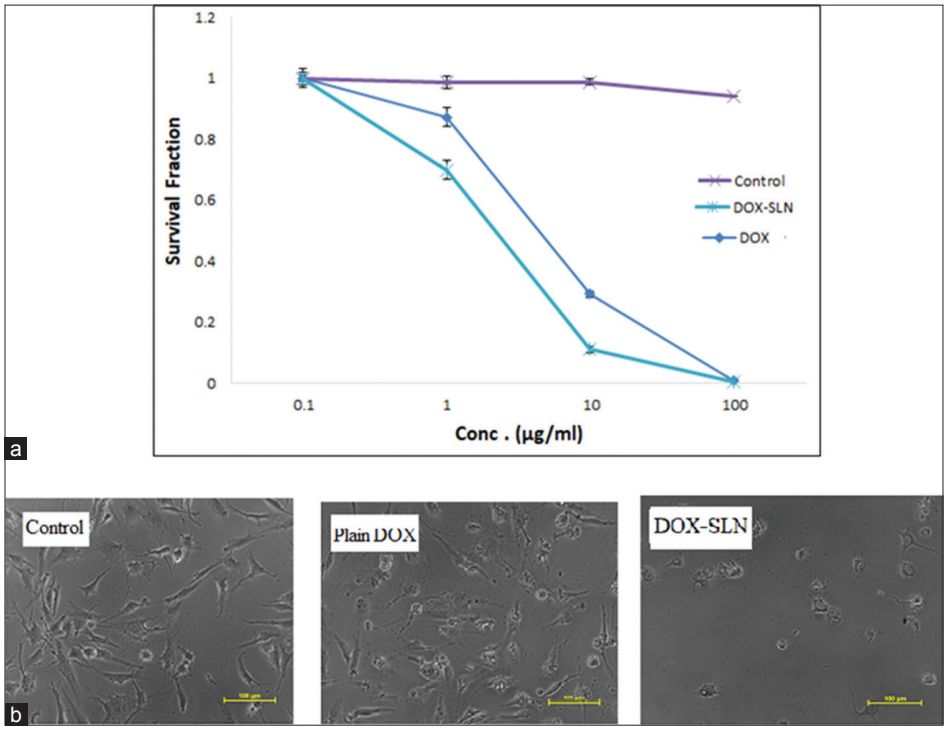

Figure 5: Cytotoxicity assessed by SRB assay on U87MG cells after 48 h. (a) Percentage survival fraction of U87MG cells treated with plain doxorubicin, FAD, and SLN-C at 0.1 µg/ml, 1 µg/ml, 10 µg/ml, and 100 µg/ml dose. Data are presented as the mean±standard deviation (SD) ($n = 3$) (b) phase-contrast photomicrograph of U87 MG cells treated with plain DOX and DOX-SLN at 10 µg/ml dose

found to be < 0.3 which showed that formulation possess uniform size distribution. The surface morphology of the DOX-SLN was observed by SEM and TEM microscopy. The photomicrographs obtained by SEM and TEM revealed the spherical shape and smooth surface of the formulation [Figure 3]. The DOX-SLN were subjected to the *in vitro* drug release studies in PBS, pH 7.4 with respect to time [Figure 4]. The result found that 68.3 ± 1.3% DOX was released from the SLN in the 48 h time interval.

The cytotoxicity of the plain DOX and DOX-SLN was carried out by sulphorhodamine blue (SRB) assay method using the U87MG cell line. Cytotoxicity of both formulations was assessed by the comparison of half maximal inhibitory concentration (IC_{50}) values. The plain DOX and DOX-SLN showed 6.5 and 3.2 µg/ml, IC_{50} value, respectively. The results displayed that the plain DOX showed less toxicity than DOX-SLN toward U87 MG cells. This may be due to the Pgp efflux of plain DOX by the cancer cells, suggesting the multiple drug resistance effect of U87MG cell lines.[17] The results also displayed that DOX-SLN was more cytotoxic than plain DOX may be due to the presence of polysorbate 80 coating as a part of the SLN structure. The polysorbate 80 causes the solubilization of the lipoidal cell membrane. As a result, fluidization of membrane takes place which enhances drug permeability across the cell membrane.[18] Furthermore, the presence of polysorbate 80 also inhibits Pgp related efflux by the cancer cells and enhanced the uptake of DOX-SLN. Polysorbate 80 also facilitated the transport of DOX-SLN across the BBB through receptor-mediated endocytosis.[7,19] For both the formulations, survival fraction of cells was found to be inversely dependent on the concentration of the drug [Figure 5a]. Figure 5b also confirmed the higher toxicity of DOX-SLN than a plain drug as the number of U87MG cells in the photomicrograph of DOX-SLN was found to be less than the plain drug. The result obtained from the study revealed that polysorbate 80 coated SLN-loaded with DOX will be a better carrier for the treatment of brain cancer.

The cellular uptake studies were also performed to confirm the internalization of polysorbate 80 coated SLNs in brain cancer cells. These studies were also performed on U87MG cells at different time intervals using C6 as a fluorescent stain. The U87MG cells were incubated with the plain C6 and C6 loaded SLN. Uptake of C6 and C6-SLN was determined after 2 h and 4 h of incubation. After 2 h of incubation, 18 ± 0.3% C6 and 45 ± 0.5% C6-SLN were internalized in U87MG cells. After 4 h of incubation, the cellular uptake was increased up to 27 ± 0.3% and 56 ± 0.2%, for C6 and C6-SLN, respectively. Figure 6A and 6B clearly displayed that the percentage of fluorescent cells was increased with time as compared to untreated control cells, suggesting the enhanced cellular uptake of C6-SLNwith time ($P \leq 0.05$).[20] Inside U87MG cells, the nucleus was localized in red color using PPI stain as shown in Figure 6B (I). Blue color fluorescence, in Figure 6B (II), indicates the localization of the C6 and C6-SLN. Figure 6B (III) is the overlay of I and II, and it

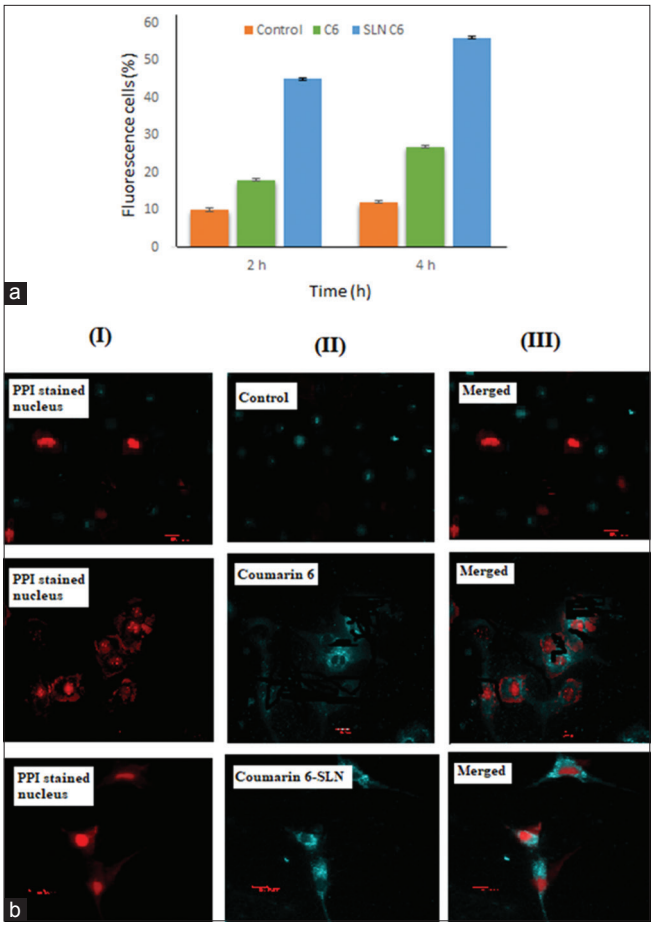

Figure 6: Confocal micrographs showing the intracellular uptake of SLNs by U87MG cells. (a) Percentage fluorescence cell after treatment with plain coumarin 6 (C6) and C6 loaded SLNs (SLN C6) after 2 h and 4 h. Data are presented as the mean±(SD)(n = 3) (B) U87MG cells incubated with C6 and SLN C6 for 2 h. The nuclei stained by propidium iodide (shown in red), and C6 and SLN C6 is intrinsically blue fluorescent.

clearly indicates that U87MG cells are highly fluorescent in case of C6-SLN than plain C6. The overlay image of C6-SLN also confirms the targeting of polysorbate 80 coated SLNs to the U87MG cells. The image clearly showed the efficiency of the optimized polysorbate 80 coated SLN in delivering the entrapped drug in U87MG cells. The results suggest the significance of polysorbate 80 coated SLN in improving the uptake of SLN inside the cancer cells.

CONCLUSIONS

The present work demonstrated the development and characterization of polysorbate 80 coated SLN for the treatment of brain cancer. The prepared SLN were characterized for the various attributes as well as cytotoxicity and cellular uptake studies on U87 MG cell lines. The SLN could stabilize the incorporated DOX and protect it from the RES uptake and Pgp related efflux. The cytotoxicity and cellular uptake studies in U87MG cell lines confirmed the

suitability of DOX loaded SLN in enhancing the cytotoxic effect of DOX against brain cancer cells. Ultimately, the results indicate that polysorbate 80 coated SLN represents a robust carrier for the delivery of drug to brain cancer cells. On the basis of the cytotoxicity and cellular uptake studies, it can be concluded that the polysorbate 80 coated SLN in comparison to plain DOX has a higher potential for the treatment of brain cancer cells.

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