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Estrogen-Anchored pH-Sensitive Liposomes as Nanomodule Designed for Site-Specific Delivery of Doxorubicin in Breast Cancer Therapy

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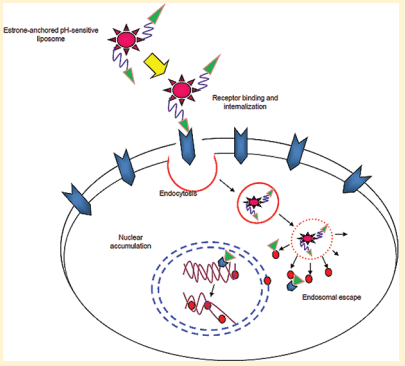
ABSTRACT: The present investigation reports the development of nanoengineered estrogen receptor (ER) targeted pH-sensitive liposome for the site-specific intracellular delivery of doxorubicin (DOX) for breast cancer therapy. Estrone, a bioligand, was anchored on the surface of pH-sensitive liposome for drug targeting to ERs. The estrone-anchored pH-sensitive liposomes (ES-pH-sensitive-SL) showed fusogenic potential at acidic pH (5.5). *In vitro* cytotoxicity studies carried out on ER-positive MCF-7 breast carcinoma cells revealed that ES-pH-sensitive-SL formulation was more cytotoxic than non-pH-sensitive targeted liposomes (ES-SL). The flow cytometry analysis confirmed significant enhanced uptake ($p < 0.05$) of ES-pH-sensitive-SL by MCF-7 cells. Intracellular delivery and nuclear localization of the DOX was confirmed by fluorescence microscopy. The mechanism for higher cytotoxicity shown by estrone-anchored pH-sensitive liposomal-DOX was elucidated using reactive oxygen species (ROS) determination. The *in vivo* biodistribution studies and antitumor activities of formulations were evaluated on tumor bearing female Balb/c mice followed by intravenous administration. The ES-pH-sensitive-SL efficiently suppressed the breast tumor growth in comparison to both ES-SL and free DOX. Serum enzyme activities such as LDH and CPK levels were assayed for the evaluation of DOX induced cardiotoxicity. The ES-pH-sensitive-SL accelerated the intracellular trafficking of encapsulated DOX, thus increasing the therapeutic efficacy. The findings support that estrone-anchored pH-sensitive liposomes could be one of the promising nanocarriers for the targeted intracellular delivery of anticancer agents to breast cancer with reduced systemic side effects.



KEYWORDS: *targeting, doxorubicin, pH-sensitive liposomes, estrogen receptors, breast cancer*

1. INTRODUCTION

Liposomes are promising carriers capable of improved systemic delivery of cytotoxic drugs including doxorubicin (DOX).[1,2] The conventional liposomal drug delivery approach suffers with the problem of accumulation of a majority of the administered dose in the reticuloendothelial system (RES). To resolve this issue, stealth liposomes have been developed, which otherwise are not subjected to rapid clearance by the RES and thus increase circulation half-life.[3] Stealth liposomal formulations such as Doxil and Caelyx have been developed and are clinically approved for the treatment of various carcinomas. However, this passive targeting approach is not fully devoid of unwanted side effects of DOX. To enhance the uptake of carrier system by tumor tissues, a ligand mediated targeting approach can be employed.[4] Ligand driven stealth liposomes may possess the ability to increase the selective delivery of DOX to the target site(s). Although ligand-receptor mediated uptake of liposomes increases the accumulation of DOX, nevertheless the rate of its intracellular release followed by endocytosis of liposomes remains slower. The incorporation of a pH-triggered release mechanism with target oriented liposomes may further improve the intracellular trafficking of DOX particularly in cancer cells, and may increase the cytotoxic potential of ligand-decorated pH-responsive liposomes.

The pH-sensitive liposomes are reported to undergo controlled fusion with endosomal membranes[5,6] followed by rapid destabilization in acidic environments such as in endosomes.[7] Basically, pH-sensitive liposomes are composed of a neutral cone-shaped lipid dioleoylphosphatidyl-ethanolamine (DOPE) and a weakly acidic amphiphile, such as cholesteryl hemisuccinate (CHEMS).[8−10] The fusogenic characteristics are due to the polymorphic phase behavior of DOPE, which does not form a bilayer structure when dispersed

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in aqueous media but forms a hexagonal structure.[11] The pH-sensitive liposomes can facilitate the cytosolic release of membrane impermeable molecules; and this property when combined with a targeting ligand may promote receptor-mediated endocytosis for targeted cytosolic delivery of bioactive(s).

The estrogen receptors (ERs) belong to nuclear hormone receptor superfamily with distribution in the tissue such as breast and uterus and amplified expression over 85% in breast carcinomas.[12,13] The liposomes incorporating estrone derivative, estrone-PEG-DSPE, have been shown to efficiently deliver DOX into ER-bearing tumor cells *via* receptor-mediated endocytosis.[2,14] These liposomes bind selectively to ERs overexpressed by the breast cancer cells triggering endocytosis of the liposomes followed by their ultimate transfer into lysosomes. Mechanistically, during these bioevents liposomes lead to very slow release of DOX in the cell cytoplasm.

In the present work, it was hypothesized that a pH-sensitive formulation with ER targeting potential could further improve intracellular delivery of DOX to the tumor cells possessing amplified levels of ERs, like breast cancer. Therefore, ER-targeted pH-sensitive liposomes encapsulating DOX were developed and characterized for the breast cancer treatment. The therapeutic efficacy of estrone-anchored pH-sensitive liposomes (ES-pH-sensitive-SL) was compared with estrone-anchored non-pH-sensitive liposomes (ES-SL). The developed system was evaluated for intracellular trafficking, cytotoxicity, cellular uptake and nuclear localization on ER-positive MCF-7 cells breast cancer cells. The mechanism of cytotoxic effect of ES-pH-sensitive-SL was also studied, to justify advantage of cytosolic delivery of DOX. Biodistribution and antitumor activity was performed on tumor bearing mice to estimate their *in vivo* potential. The cardiotoxic side effects of ES-pH-sensitive-SL were also assessed to ascertain safe and efficient targeted drug delivery.

2. MATERIALS AND METHODS

2.1. Materials. Egg phosphatidylcholine (PC), dioleoylphosphatidylethanolamine (DOPE), cholesteryl hemisuccinate (CHEMS), distearoylphosphatidylethanolamine (DSPE), 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium (MTT), Sephadex G-50, dicyclohexylcarbodiimide (DCC), dimethylaminopyridine (DMAP), hydroxybenzotriazole (HOBT), triethylamine (TEA) and Trizma base were purchased from Sigma (St. Louis, MO, USA). Estrone (ES), tissue culture media, penicillin−streptomycin, and fetal bovine serum (FBS) were obtained from Himedia (Bombay, India). Doxorubicin was provided as a gift sample by Sun Pharma (Vadodara, India). All reagents and solvents unless specified were of either analytical or HPLC grade.

2.2. Liposome Preparation. The estrone was conjugated to DSPE-PEG by a previously reported method[14] and then incorporated in the liposomes by adding DSPE-PEG−ES as lipid component. ER-targeted pH-sensitive liposomes (ES-pH-sensitive-SL) and ER-targeted non-pH-sensitive liposomes (ES-SL) were prepared by a cast film method as previously reported with slight modifications.[15] The liposomes were composed of DOPE:HSPC:CHEMS:CHOL:ES−PEG-DSPE for ES-pH-sensitive-SL and HSPC:CHOL:ES−PEG-DSPE for ES-SL at various molar ratios with ES−PEG-DSPE at 5 mol % to phospholipids. Briefly, lipids were dissolved in chloroform and solvent was evaporated to dryness under vacuum using a rotary evaporator (Strike, 102, Italy). The dried lipid films were then hydrated with appropriate buffers. The liposomes were sonicated to reduce vesicle size with a probe sonicator (Lark, India) for 5 min and were extruded 10 times through a 0.22 μm polycarbonate membrane. DOX was encapsulated into the liposomes by the ammonium sulfate gradient method.[16] The unentrapped DOX was then separated by gel exclusion chromatography on a Sephadex G-50 column eluted with HEPES buffered saline (pH 7.4). The concentration of liposome-entrapped DOX was determined by UV spectrophotometry at λ_{max} 480 nm following the disruption of vesicles by Triton X-100.

2.3. Characterization of Liposomes. *2.3.1. Size, Shape and Morphology.* The morphological examination of liposomes was performed by transmission electron microscopy (TEM) (JEOL, Japan) using negative staining (1% PTA).[17] The mean vesicle size expressed as volumetric diameter was measured by photon correlation spectroscopy (Zetasizer, ZS 90, Malvern, U.K.).

2.3.2. Entrapment Efficiency. Entrapment efficiency (EE %) was determined using the previously reported method.[18] Briefly, 0.2 mL of liposome dispersion was eluted with PBS (pH 7.4) through a Sephadex G-50 column to separate free drug. The entrapped drug was determined by disrupting the vesicles with Triton X-100 and measuring absorbance at 480 nm by UV-spectrophotometer (Cintra10, Japan). Entrapment efficiency was determined by the ratio of the actual amount of encapsulated drug over the total amount of drug used for the preparation of liposomes.

2.3.3. In Vitro Drug Release. The *in vitro* release pattern of DOX was investigated with buffers at pH 7.4 and pH 5.5, using the dialysis tube method. Briefly, 1 mL of liposomal suspension was taken into a dialysis tube [MWCO: 10,000, (Sigma MO, USA)] and introduced into the release medium. The temperature of the assembly was maintained at 37 ± 1 °C. Samples were withdrawn at predetermined time intervals and replaced with the same volume of release medium. The withdrawn samples were assayed for drug content by measuring absorbance at 480 nm.

2.3.4. pH Dependent Change in Zeta Potential. The zeta potential of the resulting liposomes at various pH values was measured with a Zetasizer. The liposome dispersion in 20 mM acetate buffer (pH 4.0, 4.5, 5.0 5.5, 6.0, 6.5 and 7.0) or 20 mM PBS (pH 7.4) was taken and measured three times at 37 ± 1 °C.

2.3.5. pH Induced Liposomal Aggregation. Liposomal aggregation in response to pH change was measured by increase in vesicle size. Some 40 μL sample of the liposomes was diluted in 4 mL of acetate buffer of various pH values and incubated at 37 ± 1 °C. At various time points, aliquots of the samples were withdrawn and the mean particle diameter was determined.

2.4. Cell Culture. The ER-positive human breast cancer cells (MCF-7) were selected for the study. The cell lines were cultured in RPMI supplemented with 2 mM glutamine hydrocortisone, 1% streptomycin/penicillin and 10% fetal calf serum, maintained in a humidified atmosphere containing 5% CO_2 at 37 ± 1 °C.

2.5. DOX Uptake Study. *2.5.1. Microscopy.* Cells were plated at a density of 0.1 × 10^6 cells/well containing 12 mm sterile coverslips for 24 h. Free DOX, ES-SL and ES-pH-sensitive-SL with equivalent DOX concentration (10 μM) were incubated for 3−24 h at 37 ± 1 °C in 5% CO_2/95% air. After defined time intervals, the cells were washed 2 times with PBS

and immediately observed in green light under the fluorescence microscope (Olympus 1×70).

2.5.2. Nuclear Localization. Twenty-four hours before incubation with liposomes, cells were seeded at 0.5×10^6 per 12 mm glass coverslip. Liposomal DOX formulations (10 μM) were incubated for 3−6 h at 37 ± 1 °C and 5% CO_2 in a humidified environment. Cells were then placed on ice, washed three times with ice cold PBS, fixed with 90% alcohol for 20 min and counterstained with (10 μM) DAPI. Cells were imaged on an Olympus 1×70 fluorescent microscope.

2.5.3. Flow-Cytometric Analysis. Cells were seeded at a density of 0.1×10^6 cells/well for 24 h. Treatments were carried out with free DOX and liposomal DOX formulations (10 μM) each for 3−24 h at 37 ± 1 °C in 5% CO_2/95% air and cells were washed twice with PBS and collected through centrifugation (1000 rpm for 10 min). Cells were resuspended in PBS before measurements. The cells were fixed with 90% alcohol, before analysis. Fluorescence histograms were recorded with a BD FACS Calibur (Beckton Dickinson) flow cytometer and analyzed using Cell Quest software. Minimums of 10,000 events were analyzed to generate each histogram. The gate was arbitrarily set for the detection of green fluorescence (150, FL1-H, 400, 0, FSC, 200, linear scale).

2.5.4. Competitive Inhibition of Liposomal DOX in Presence of Free Estrone. Cells (0.1×10^6) per well in serum free medium were incubated with DOX and liposomal DOX (10 μM) together with free estrone (2 mg/mL) for 1 h at 37 ± 1 °C. Cells were washed twice with PBS and collected in the tube by centrifugation (1000 rpm for 10 min). Cells were resuspended in PBS before measurements. Fluorescence histograms were recorded with a BD FACS Calibur (Beckton Dickinson) flow cytometer and analyzed using Cell Quest software. Minimums of 10,000 events were analyzed to generate each histogram. The gate was arbitrarily set for the detection of green fluorescence (150, FL1-H, 400, 0, FSC, 200, linear scale).

2.5.5. Reactive Oxygen Species (ROS) Measurement. Cells were seeded at a density of 0.5×10^6 cells per well for 24 h and incubated with free DOX, ES-SL-DOX and ES-pH-sensitive-SL-DOX (10 μM) for 6 and 24 h at 37 ± 1 °C in 5% CO_2/95% air. After the incubation, cells were washed with PBS and incubated for 30 min with 20 μM dichlorofluorescein diacetate (DCFH-DA) dye. Cells were collected in the tube by centrifugation (1000 rpm for 10 min) and resuspended in PBS. Fluorescence histograms were recorded with a BD FACS Calibur (Beckton Dickinson) flow cytometer and analyzed using Cell Quest software. Minimums of 10,000 events were analyzed to generate each histogram. The gate was arbitrarily set for the detection of green fluorescence (150, FL1-H, 400, 0, FSC, 200, linear scale).

2.6. Cytotoxicity Studies. The cytotoxic effect of liposomes was determined by MTT assay on MCF-7 cells. These cells were seeded in a 96 well plate at a density of 0.5×10^5 cells/well. After the cells formed a monolayer, culture medium was replaced with 200 μL of medium containing serial dilutions of DOX formulations. Samples were cultured in six wells, and the experiment was done in triplicate. After incubation at 37 ± 1 °C for 72 h, the wells were washed gently with PBS (pH 7.4). One hundred microliters of MTT solution (0.5 mg/mL) was then added to each well and incubated for 4 h at 37 ± 1 °C. After removal of culture solution, the precipitant was dissolved in 100 μL of isopropanol. Optical densities were measured at 540 nm using a series 750 microplate reader (BIO-RAD, model 680, U.K.). Absorbance was directly related to % cell viability, and control cell viability was considered 100% at each respective time point. The 50% inhibitory concentration (IC_{50}) was determined as the drug concentration which resulted in a 50% reduction in cell viability.

2.7. *In Vivo* Studies. The *in vivo* studies were performed in female Balb/c mice. The studies were carried out as per the guidelines compiled by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animal, Ministry of Culture, Government of India). All the study protocols were approved by the Institutional Animal Ethics Committee.

2.7.1. Biodistribution Study. The biodistribution studies were performed on tumor bearing female Balb/c mice. DMBA (7,12-dimethylbenz[*a*]anthracene) was administered orally (soybean oil) to mice in three doses of 45 mg/kg dose at weekly intervals to generate the tumor. The treatment consists of four groups and thirty-six mice in each group. The first group received free DOX solution in PBS (pH 7.4) administered intravenously at 5 mg/kg body weight through tail vein injection. The second group and third group received ES-SL and ES-pH-sensitive-SL, respectively. The fourth group received PBS (pH 7.4) alone and served as control. The animals were anesthetized and sacrificed at 1, 3, 5, 8, 24, and 48 h for the collection of visceral organs like heart, liver, kidney and tumor immediately. The organs were washed with Ringer's solution to remove any adhered debris and dried using a tissue paper. The tissues were homogenized in 0.8 mL of citric acid buffer (pH 6.0) for 3 min at a speed of 3000 rpm. The homogenate was transferred to a centrifuge tube, and 3.0 mL of a mixture of chloroform and methanol (1/4, v/v) was added to the same tube, shaken using a vortex mixer for 1 min and centrifuged at 2500 rpm for 15 min. After centrifugation, 3.0 mL of the chloroform layer was transferred to another tube and evaporated to dryness under nitrogen gas in a bath at 50 °C. The residues were dissolved in 200 μL of methanol and injected into an HPLC system. The detection wavelength was 254 nm, and the mobile phase consisted of acetonitrile−water (32/68, v/v, pH adjusted to 3.0 by phosphoric acid). The flow rate was maintained at 1.5 mL/min, and the analytical column was of C_{18} (4.6 × 250 mm, 5 μm).[19]

2.8. *In Vivo* Evaluation of Antitumor Efficacy. The *in vivo* therapeutic efficacy of the liposomal DOX was assessed in DMBA induced breast tumor bearing female Balb/c (four-week-old, 12−14 g) mice. Drug treatment was given after 10 weeks of the last dose of DMBA. The tumor width (W) and length (L) were recorded with a caliper, and tumor size was calculated using the formula (tumor size) = $LW^2/2$. After 10 weeks of tumor induction, tumor bearing animals were separated and divided randomly into different treatment groups. Animals were treated with a single dose of 5 mg of DOX/kg body weight (group A), ES-SL and ES-pH-sensitive-SL with equivalent dose of DOX (groups B and C). The control group received a 250 μL intravenous injection of PBS (pH 7.4). The tumor size was calculated by the above formula. The study was terminated after 30 days post treatment.

2.9. Assessment of Cardiotoxicity. Female Balb/c mice were used for determination of cardiotoxicity. Free DOX, ES-SL and ES-pH-sensitive-SL were administrated by iv route at a single dose (5 mg/kg body weight). Blood samples were drawn from the retro orbital plexus under light ether anesthesia, into nonheparinized capillary tubes on day 21 after iv injection of formulations. Serum was separated by centrifugation at 4000 rpm for 20 min and stored at −20 °C until further analysis.

Serum enzyme activities such as LDH (lactate dehydrogenase) and CPK (creatine phosphokinase) levels were assayed using commercially available kits, LDH and CK-MB (Crest Biosystem, India). The toxicity of DOX on cardiac cells was assessed by level of LDH and CPK enzymes released from heart.

2.10. Statistical Analysis. All data were expressed as mean ± SD. Comparisons among three or more groups were performed by analysis of variance (ANOVA) followed by post hoc Tukey–Kramer test. For comparison between two groups, Student's *t* test was applied. A p value less than 0.05 (i.e., $p < 0.05$) was considered to indicate statistical significance for all comparisons.

3. RESULTS

3.1. Preparation and Characterization of Estrone-Anchored pH-Sensitive Liposomes. ES-pH-sensitive-SL were prepared by incorporating estrone modified lipid, i.e., ES–PEG-DSPE with other lipids such as DOPE, HSPC and CHEMS. The ES–PEG-DSPE conjugate was synthesized by a previously reported method with minor modifications[14] and was characterized by IR spectroscopy. The DOX was loaded by a transmembrane pH gradient method.[20] The average vesicle size of ES-pH-sensitive-SL and ES-SL was found to be 151 ± 59 nm and 138 ± 43 nm, respectively (Table 1). The DOX/lipid ratio and encapsulation efficiency were almost equivalent among the investigated liposomes. TEM observations of these liposomes demonstrated the spherical nature of liposomes (Figure 1). Furthermore, *in vitro* release of DOX from liposomes was investigated in different pH buffer mediums. Figure 2a shows that the release of DOX from ES-pH-sensitive liposomes was higher (90%) at low pH 5.5 within 2 h (corresponding to the pH of endosome), however, it was only 40% at pH 7.4 after 24 h (corresponding to the pH of blood). However, the release of DOX from ES-SL was approximately same, i.e., about 30% after 24 h (Figure 2b).

Table 1. Size, Entrapment Efficiency and Polydispersity Index (PDI) of Different Liposomal Formulations[a]

formulation code	size (nm)	entrapment effic (%)	PDI
ES-SL	138 ± 43	91 ± 2.3	0.0234
ES-pH-sensitive-SL	151 ± 59	90 ± 7.6	0.0347

[a]Data represent mean ± SD (n = 3).

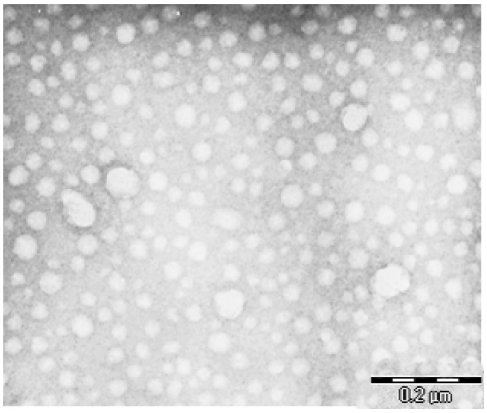

Figure 1. TEM image of ES-pH-sensitive-SL.

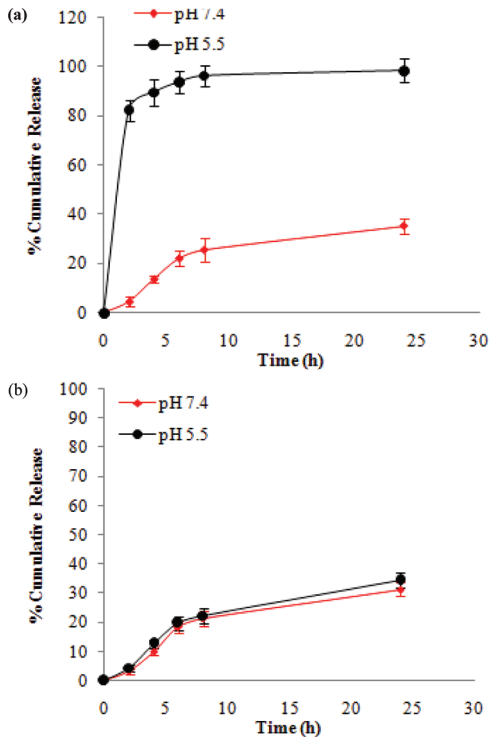

Figure 2. *In vitro* release behavior of DOX at different pH conditions: (a) ES-pH-sensitive-SL; (b) ES-SL. Data represent mean ± SD (n = 3).

3.2. pH Dependent Aggregation and Zeta Potential. The fusogenicity of the developed formulations was estimated by pH dependent change in vesicle size and zeta potential. As shown in Figure 3a, the mean diameter of the ES-pH-sensitive-SL was significantly increased at low pH, indicating vesicle aggregation and/or membrane fusion. The time dependent liposome aggregation at pH 5.5 is shown in Figure 3b. The mean vesicle size of ES-pH-sensitive-SL became progressively larger as pH was decreased, but this increased vesicle size could not be reversed by adjusting the pH back to 7.4. At pH 5.5, vesicle size of ES-pH-sensitive-SL increased rapidly within the 10 min of incubation; however, prolonged incubation showed a slower rate of further increase in vesicle size. At the same time ES-SL did not show any pH dependent fusion action or increase in vesicle size. The zeta potential of the formulations was measured in various pH buffer conditions. It was observed that the zeta potential of ES-pH-sensitive-SL was shifted from +35 mV to −24 mV when pH value was changed from 4.5 to 7.4. In addition, the pH values where the zeta potential of these liposomes became zero were around 5.0. By contrast, ES-SL showed a constant zeta potential at all pH values studied.

3.3. Analysis of DOX Uptake by Fluorescence Microscopy. The internalization of DOX in MCF-7 cells was studied using fluorescence microscopy by tracking its autofluorescence. Equivalent amounts (10 μM) of free DOX, ES-SL-DOX and ES-pH-sensitive-SL-DOX were added to MCF-7 cells for 3–24 h. The Figure 4 showed that in all cases DOX gains access to the cell nucleus, but the kinetics and apparently the route of uptake were different in all cases. Intracellular distribution of ES-SL and ES-pH-sensitive-SL was quite different from that of free DOX. After 3 h of incubation with free DOX, strong fluorescence was observed in cell nuclei in addition to a very weak fluorescence in the cytoplasm,

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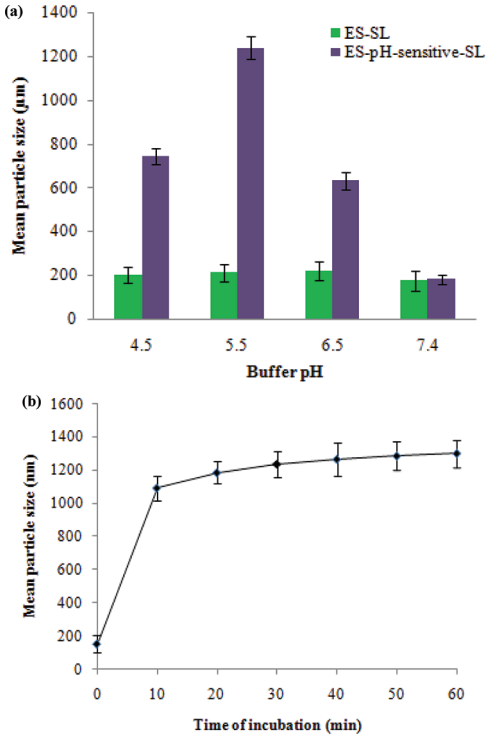

Figure 3. pH dependent aggregation of ES-pH-sensitive-SL: (a) he mean diameter of the liposomes following a 30 min incubation at 37 °C at various values of buffer pH; (b) time dependent increase in liposome size at pH 5.5. Data represent mean ± SD (n = 3).

suggesting rapid internalization of DOX to the nucleus after passive diffusion into the cells (Figure 4C1). In the case of ES-SL, DOX was largely localized in the endosomal compartment, demonstrating receptor mediated endocytosis (Figure 4B1). In the case of ES-pH-sensitive-SL, DOX fluorescence exhibited speckled red dots throughout the cytoplasm (Figure 4A1), indicating that ES-pH-sensitive-SL were initially trapped within endosomal compartments which then rapidly released DOX in the cytosol by pH dependent endosome fusion as suggested by *in vitro* release study. After 6 h of incubation in the case of ES-pH-sensitive-SL, DOX fluorescence was found to be very strong mainly in the nuclei and spackled fluorescence in the cytosol; whereas a weak fluorescence was observed in the nuclei in the case of ES-SL (Figures 4A2 and 4B2). After 24 h, DOX fluorescence was observed only in the cells that were exposed to ES-SL and ES-pH-sensitive-SL, which was very much less in the case of free DOX (Figures 4A3, 4B3 and 4C3).

3.4. Nuclear Localization. The distribution pattern of DOX was further studied to determine selective nuclear localization of ES-anchored-pH-sensitive-SL. Figure 5 shows localization of DOX, after 3 h of treatment followed by fixation and DAPI counterstaining. Very small DOX signal was detected after 3 h of incubation with ES-SL (Figure 5B1) by colocalization of DOX and DAPI; however, after 6 h strong fluorescence was observed in the nucleus (Figure 5B1′). The ES-pH-sensitive-SL showed strong fluorescence in the intracellular compartment after 3 h of incubation (Figure 5A1) and in the nucleus after 6 h (Figure 5A1′). In contrast to liposomal-DOX, free DOX demonstrated higher accumulation in the nucleus after 3 h (Figure 5C1) and slight fluorescence was observed in the nucleus after 6 h (Figure 5C1′).

3.5. Cell Uptake by Flow Cytometry. Flow cytometric analysis was carried out for quantitative determination of DOX uptake by MCF-7 cells. The fluorescent intensity was directly proportional to the amount of DOX internalized. The DOX fluorescence within the cells at different time points was

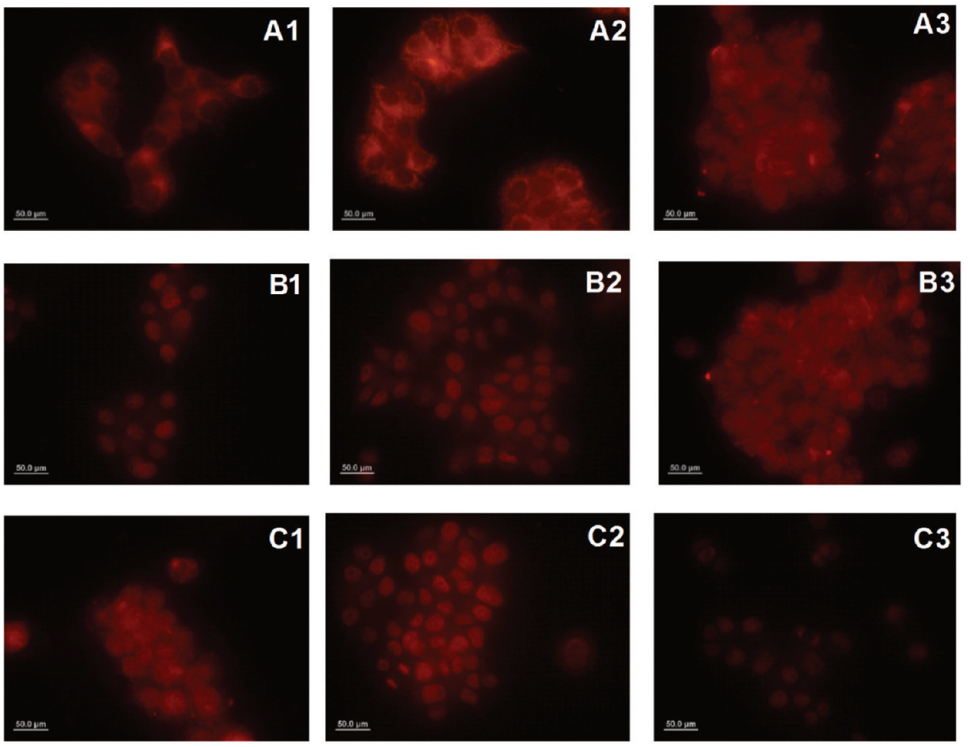

Figure 4. Fluorescent images of breast cancer MCF-7 cells incubated with ES-pH-sensitive-SL (A), ES-SL (B) and free DOX (C) for 3, 6, and 24 h at 37 °C. All three DOX formulations have equivalent concentrations (10 μM). (Magnification 40×.)

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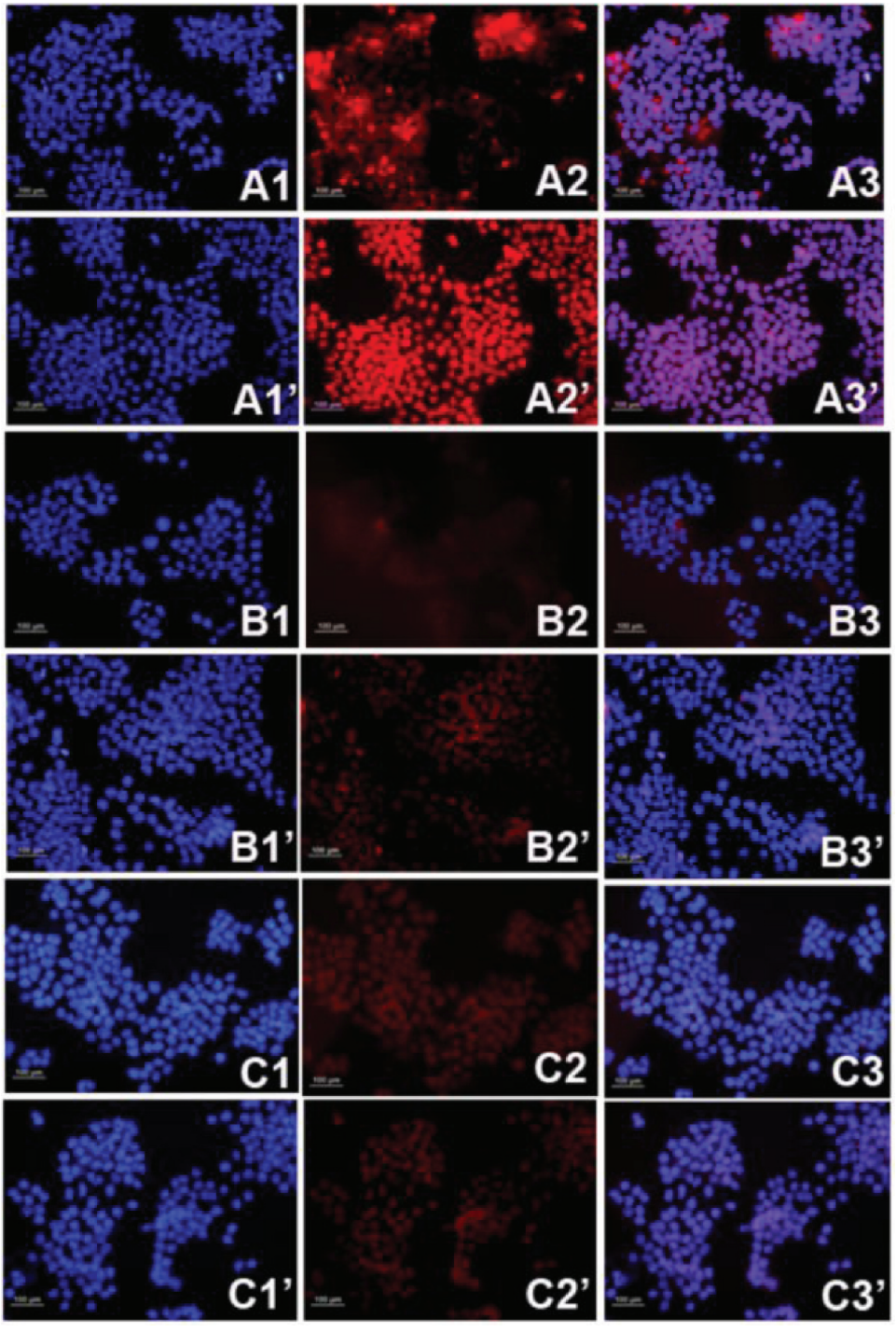

Figure 5. Nuclear localization of DOX: fluorescence microscopy images of MCF-7 cells treated with ES-pH-sensitive-SL (A), ES-SL (B) and free DOX (C) showing DOX localization at 3 and 6 h incubation times. Blue-fluorescence of DAPI (nuclear staining marked by 1), red-fluorescence of DOX (marked by 2) and merged images showing colocalization of DAPI and DOX (marked by 3) for respective cells. (Magnification 40×.)

recorded and compared with negative control. The results demonstrated that both the ER-targeted liposomes (pH sensitive as well as non-pH-sensitive) containing DOX had a significantly greater uptake ($p < 0.001$) than the free DOX (Figure 6). However, the uptake of ES-pH-sensitive-SL was significantly greater ($p < 0.05$) in comparison to ES-SL, resulting in higher intracellular accumulation of DOX. The competitive inhibition experiment was carried out in the presence of free estrone in the incubating media. Addition of free estrone significantly ($p < 0.001$) reduced mean fluorescence intensity of both the ES-targeted liposomes compared to free DOX (Figure 6).

3.6. Reactive Oxygen Species (ROS) Generation. To find out the role of ROS induced cytotoxicity in free DOX and DOX loaded liposome treated cells, the ROS level at 6 and 24 h time points was determined (Figure 7).[21,22] After various treatments the ROS level was increased up to 2.72-, 2.04-, and 1.4-fold in the case of ES-pH-sensitive-SL, ES-SL and free DOX when compared with the control cells without any treatment. There was no DCF fluorescence observed in the case of

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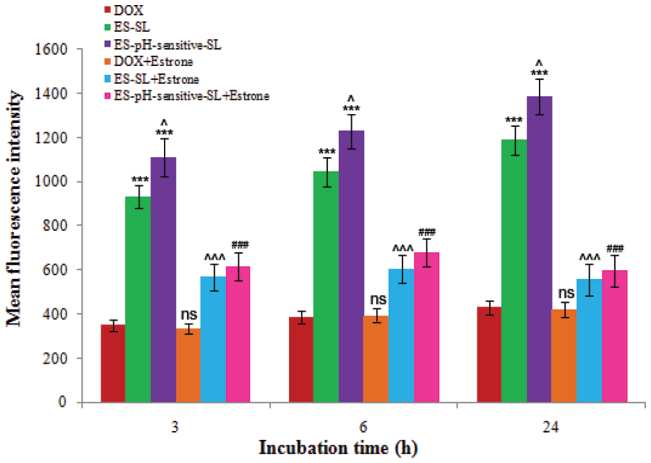

Figure 6. Uptake of DOX and liposomal DOX formulations by MCF-7 cells. $^{***}p < 0.001$ when compared with free DOX, $^{\wedge}p < 0.001$ when compared with ES-SL and $^{\#\#\#}p < 0.001$ when compared with ES-pH-SL. Data represent mean ± SD ($n = 3$).

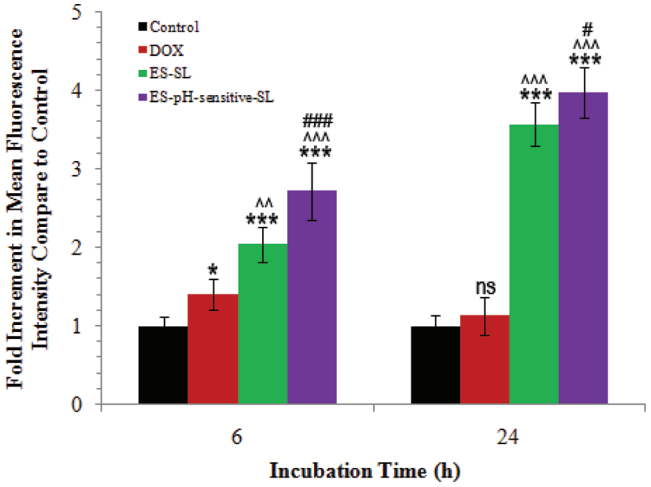

Figure 7. ROS level in MCF-7 cells compared to control after treatment with DOX, ES-SL and ES-pH-sensitive-SL at 6 and 24 h at equivalent DOX concentration (10 μM). Data represent mean ± SD ($n = 3$). $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ when compared with control, $^{\wedge}p < 0.001$, $^{\wedge}p < 0.01$, $^{\wedge}p < 0.05$ when compared with free DOX, and $^{\#\#\#}p < 0.001$, $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ when compared with ES-SL, ns means nonsignificant.

untreated cells even after 24 h. The DOX mediated ROS level was significantly ($p < 0.05$) higher in comparison to the cells without any treatment. This may be due to well-known induction of ROS generation by DOX.[23]

3.7. Cytotoxicity Studies. *In vitro* cytotoxicity of the formulation was estimated using MTT assay. The cytotoxicity of ES-pH-sensitive-SL was compared with ES-SL and free DOX. Results showed that, at all concentrations of DOX, ES-pH-sensitive-SL was more cytotoxic than ES-SL and the cytotoxic efficiency of ES-pH-sensitive-SL was almost comparable to that of the free DOX, since DOX is an amphipathic molecule that can easily cross the cell membrane, thus demonstrating the higher cytotoxic efficiency than formulations. Similarly, in the case of ES-pH-sensitive-SL, fast release of DOX from endosome by a pH dependent mechanism resulted in higher cytotoxicity than ES-SL. The ES-pH-sensitive-SL had significantly lower IC_{50} than ES-SL ($p < 0.05$) (Table 2). The cell growth curve of DOX after incubation with different formulations is presented in Figure 8.

Table 2. IC_{50} Values of Different DOX Formulations[a]

formulation code	IC_{50} (μM DOX) MCF-7 cells
ES-pH-sensitive-SL	0.8 ± 0.2
ES-SL	3.3 ± 0.7
DOX	0.15 ± 0.03

[a]Data represent mean ± SD ($n = 3$).

3.8. *In Vivo* Biodistribution. *In vivo* biodistribution was performed on tumor bearing female Balb/c mice. Both the liposomal formulations, i.e., ES-SL and ES-pH-sensitive-SL, showed prolonged systemic circulation in blood after iv administration. However, ES-pH-sensitive-SL was cleared rapidly as compared to ES-SL (Figure 9).

The level of DOX in different tissues after iv administration of ES-pH-sensitive-SL, ES-SL and free DOX at a dose of 5 mg/kg is shown in Figure 10. The DOX contents in tumor tissue was higher for the ES-SL ($^{***}P < 0.001$) and ES-pH-sensitive-SL ($^{***}P < 0.001$) compared to that of free DOX at all times. However, among the two liposomal formulation, ES-pH-sensitive-SL formulation accumulated significantly more in tumor ($^{\wedge}P < 0.001$) than ES-SL (Figure 10A). Cardiotoxicity is the main drawback of doxorubicin therapy. The DOX concentration of the ES-pH-sensitive-SL in the heart was significantly lower than those of free DOX ($^{***}P < 0.001$) and the ES-SL ($^{\wedge}P < 0.05$) (Figure 10B) after 3 h . The concentrations of DOX in the liver tissues with liposomal formulations were significantly lower than those of free DOX ($^{***}P < 0.001$) (Figure 10C). In the kidney DOX concentrations of the ES-pH-sensitive and ES-SL were significantly lower than those of the free DOX ($^{***}P < 0.001$) (Figure 10D).

3.9. Antitumor Activity. Antitumor activities of the different DOX formulations were evaluated in DMBA induced breast cancer model after a single iv administration (5 mg of DOX/kg body weight). The free DOX, ES-SL and ES-pH-sensitive-SL significantly suppressed tumor growth as compared to control (Figure 11). However, among all ES-pH-sensitive-SL showed higher tumor suppression than other formulations.

3.10. Assessment of Cardiotoxicity. The clinical side effect of DOX is cardiotoxicity, which limits its wide applicability. The cardiotoxic potential of developed formulations was measured by the production of specific enzymes, mainly LDH and CPK. The levels of LDH and CPK enzymes in serum were lower when DOX was encapsulated in the liposomes, as compared to free DOX (Figure 12).

4. DISCUSSION

The selectivity of estrone-anchored liposomes for breast cancer targeting has been previously reported.[14] The developed system successfully increased the therapeutic index of DOX against breast cancer. In the present study, we have attempted to further increase the efficacy of ES-targeted-liposomal formulation of DOX by increasing the rate of intracellular drug release employing pH-sensitive targeted liposomes. Combinatorial effects of targeting potential of estrone and intracellular delivery potential of the pH-sensitive liposomes were tested using the antineoplastic agent DOX. It was expected that the estrone-modified pH-sensitive liposomes can enhance the

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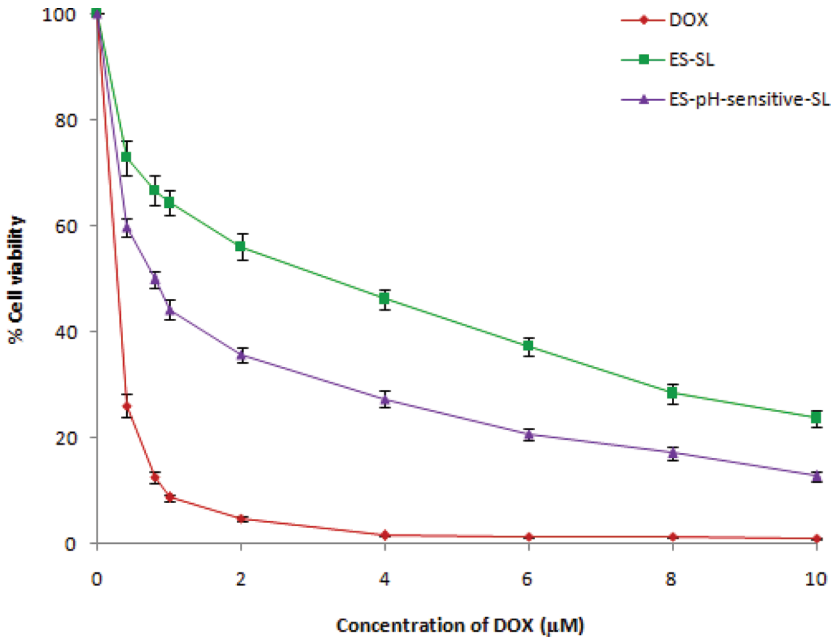

Figure 8. Cell growth curve of DOX after incubation (72 h) with free DOX, ES-SL and ES-pH-sensitive-SL formulation. (n = 3).

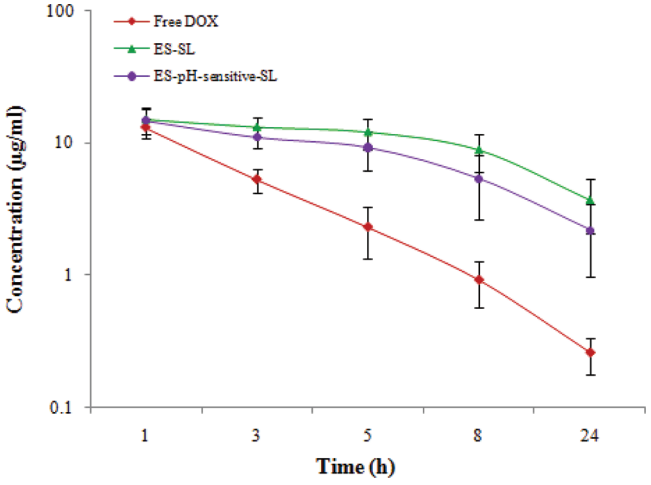

Figure 9. Plasma profile of different DOX formulations. Data represent mean ± SD (n = 6).

intracellular uptake of the DOX to ER-positive breast cancer cells and hence may lead to improved antitumor activity.

The ES-pH-sensitive liposomes and ES-SL were prepared by incorporating estrone modified lipid with DOPE and cholesteryl hemisuccinate, that impart targeting as well as pH-sensitive properties to the liposomal system. The *in vitro* release profile indicated the stability of vesicles during circulation and their ability to release the DOX under acidic conditions. The so observed pattern may be due to incorporation of DOPE and CHEMS within the liposomal membrane. The DOPE is reported to be taken up in a hexagonal structure in an aqueous medium at neutral pH.[24] But, when combined with stabilizing components such as cholesteryl hemisuccinate (CHEMS), it can assemble into bilayers. Therefore, such liposomes are assumed to destabilize in the acidic environment, rapidly releasing their contents[25] due to protonation of CHEMS at low pH (<6.0), which accelerates the destabilization of DOPE vesicles by promoting the conversion of bilayers into the hexagonal phase.[24] The fusogenic potential of the pH-sensitive liposomes is the most important property for releasing encapsulated content from the endosome into the cytosol. The ES-pH-sensitive-liposomes showed irreversible change in vesicle size with the decrease in pH, suggesting the membrane fusion of vesicles, while ES-SL showed no pH dependent fusion. The developed ES-pH-sensitive-SL also changed their zeta potential according to the surrounding buffer pH, further confirming pH-responsive behavior of the formulations that can show cytosolic release of content due to endosomal escape.

The fluorescent microscopy data strongly demonstrated that ES-anchored-pH-sensitive-SL served as an efficient vehicle to transport DOX into the cytosol and suggested the different internalization mechanisms of various formulations. Both the targeted liposomes were taken up *via* receptor mediated endocytic pathway and were then localized in acidic endocytic compartments, i.e., endosomes. The ES-SL was transported to lysosome after endocytosis and subsequently attacked by lysosomal digestive enzymes, resulting in liberation of DOX into the cytosol. Subsequently, DOX penetrates through the nuclear membrane to reach the nucleus. Despite there being no difference in cellular uptake of ES-SL and ES-pH-sensitive-SL, DOX release from endosomes was significantly improved by employing pH-sensitivity characteristics. The ES-pH-sensitive-SL was internalized by receptor mediated endocytosis, and subsequently, fusion of liposomes with endosomal membrane in the acidic milieu of endosome results in triggered release of entrapped DOX in the cytosol. In the acidic environment of endosomes, the net charge of ES-pH-sensitive-SL switches to being positive, thus fusion with endosomal membrane facilitated the intracellular accumulation of DOX.

Three hours after the addition of ES-SL, a large number of DOX-liposomes were endocytosed and transferred to lysosomes.[25,26] Subsequent attack by the lysosomal enzymes resulted in the liberation of DOX into the cytosol from where it reaches to the nucleus (Figure 5B2). By contrast, ES-pH-sensitive-SL facilitates DOX release by a different mechanism within 3 h (Figure 5A1). Upon endocytosis of these liposomes, fusion occurs with the endosomal membrane

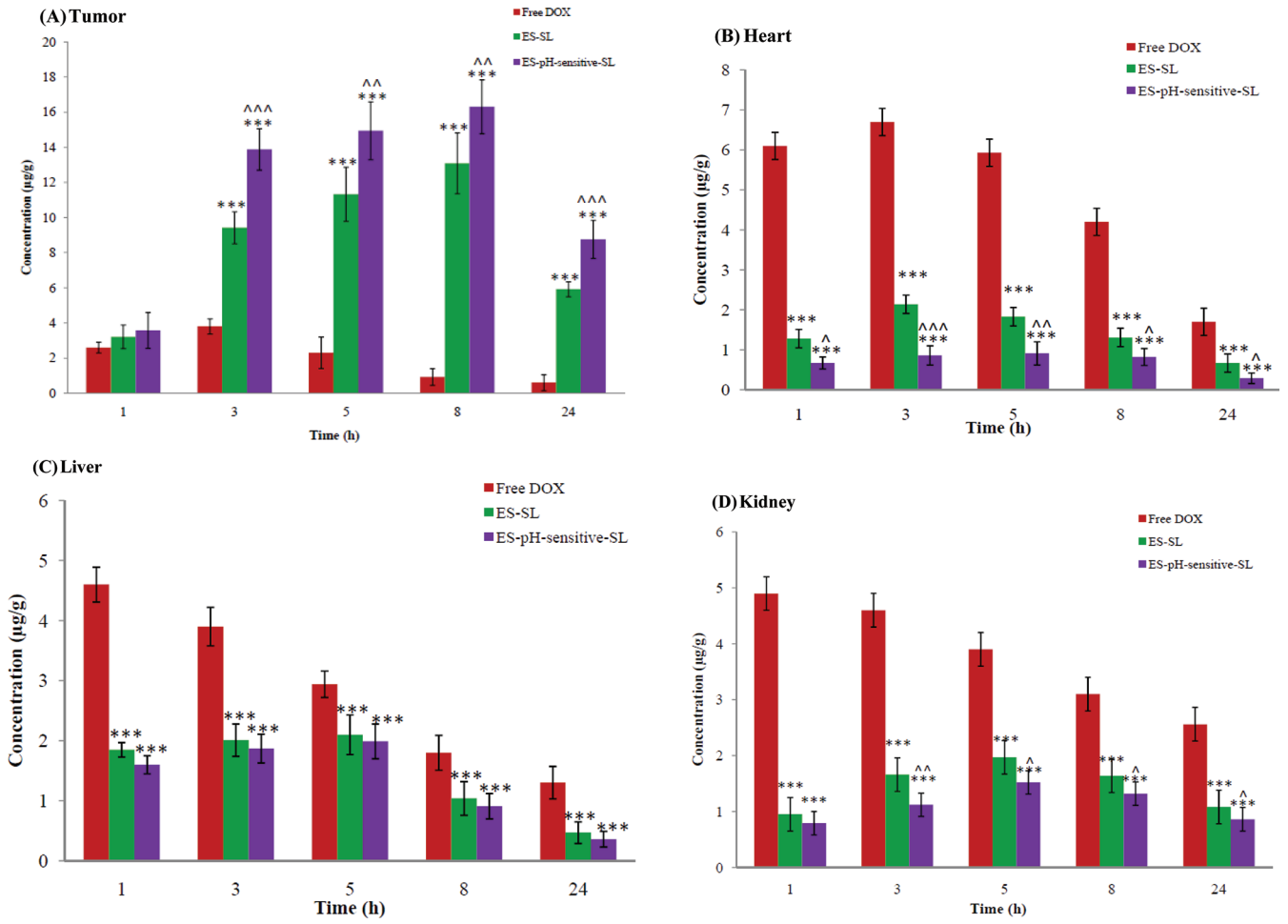

Figure 10. DOX level in various organs after iv administration of free DOX, ES-SL and ES-pH-sensitive-SL to tumor bearing mice at a dose of 5 mg/kg: (A) tumor, (B) heart, (C) liver and (D) kidney. Data represent mean ± SD (n = 6).^p < 0.001,^p < 0.01,^p < 0.05 when compared with ES-SL and ***p < 0.001, **p < 0.01, *p < 0.05 when compared with free DOX; ns means nonsignificant.

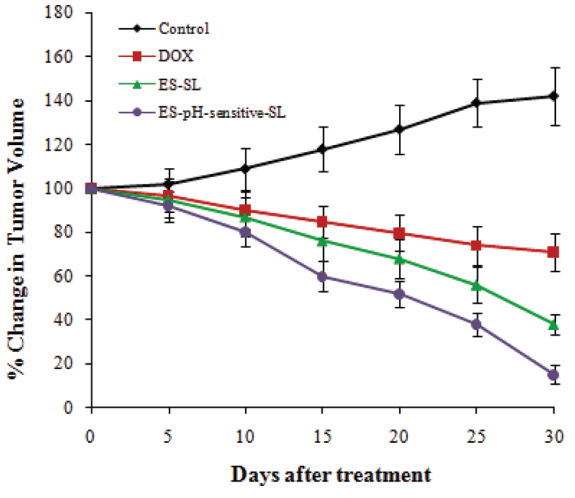

Figure 11. Tumor progression after single administration of free DOX, ES-SL and ES-pH-sensitive-SL (5 mg/kg). Tumor volume was taken as 100% at the start of drug treatment and tumor progression monitored until the end of the study. Data represent mean ± SD (n = 6).

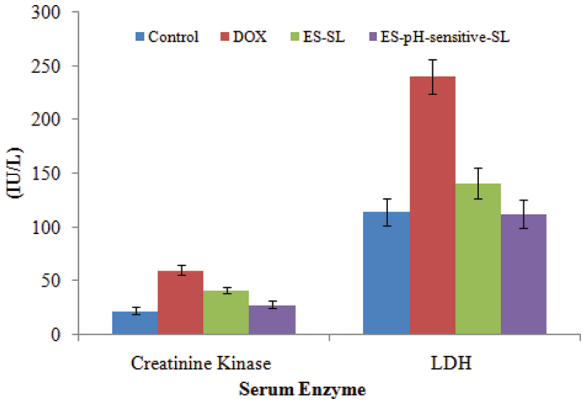

Figure 12. Serum creatine kinase and lactate dehydrogenase (LDH) levels after iv administration of free DOX, ES-SL and ES-pH-sensitive-SL. Data represent mean ± SD (n = 6).

where pH is about 4−6. Under these conditions, the net charge of the ES-pH-sensitive-SL probably switches to being positive, which facilitates fusion or aggregation of the ES-pH-sensitive-SL to endosomal membrane, resulting in DOX accumulation at the cytosol. Hence, it can be concluded that the ES-pH-sensitive-SL dramatically enhanced the DOX release from the endosomes and subsequent accumulation in the nucleus.

The DOX fluorescence intensity in the MCF-7 cells at different time demonstrated that both the ER-targeted liposomes containing DOX had a significantly greater uptake (p < 0.001) than the free DOX. This noticeable difference

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suggested that the higher DOX concentration in the ER-targeted formulations can be a result of higher ER expression level in MCF-7 cells. To further confirm receptor mediated uptake of formulation, a competitive inhibition experiment was performed by adding free estrone in the incubating media to block ERs on the surface of MCF-7 cells. Addition of free estrone significantly ($p < 0.001$) reduced mean fluorescence intensity of cells in the case of both the ES-targeted liposomes compared to free DOX (Figure 6). These results confirmed the role of estrogen receptor mediated endocytosis in efficient intracellular delivery of the DOX *via* ES-targeted liposomes.

DOX is a well-known anticancer agent for which several mechanisms of action have been reported.[27−30] Some reports suggested that doxorubicin induced calcium release from internal stores, leading to generation of ROS that can cause cell death.[28] ROS are oxygen-containing oxidizing species that are generated during cellular metabolism[29] and possess higher reactivity than molecular oxygen that can damage DNA, proteins, and lipids.[30] The intracellular level of peroxide was determined by 2,7-dichlorofluorescein diacetate (DCFH-DA).[23] The DCFH-DA is a nonfluorescent compound that permeates cells freely, where it is de-esterified to DCFH. Peroxides generated inside the cells interact with DCFH to give rise to a DCF that is fluorescent and can be analyzed by flow cytometer. This is a specific procedure for the detection of intracellular peroxides, however, it is one of the widely used assays for measuring oxidative stress in cells due to ROS.[31]

Fluorescence microscopy study (Figure 5) showed that the nuclear localization of DOX in the case of ES-pH-sensitive-SL in MCF-7 cells was much lower than that for cells incubated with free DOX, suggesting that cytotoxicity due to DNA damage will be a less prominent action of DOX in the case of ES-pH-sensitive-SL. However, the observed toxicity in ES-pH-sensitive-SL-DOX incubated cells was interestingly more than that of the free DOX treated cells. Surprisingly, ES-pH-sensitive-SL-DOX showed significantly increased ($p < 0.001$) ROS level in the cells compared to ES-SL-DOX and free DOX at 24 h (Figure 7). Therefore, predictable cell death mechanism of ES-pH-sensitive-SL-DOX could be the higher accumulation of the DOX in intracellular compartments after receptor mediated endocytosis, where the released DOX generated ROS leading to the cell death.

To study the effect of DOX-encapsulating liposomes on cellular growth of MCF-7 cells, free DOX, ES-SL and ES-pH-sensitive-SL were applied to the cells and then cellular growth was investigated. The ES-pH-sensitive-SL was more cytotoxic than ES-SL, possibly because of the overall higher release rate of DOX from ES-pH-sensitive-SL than the release rate of DOX from ES-SL. The ES-pH-sensitive-SL had IC_{50} comparable to those for free DOX (Table 2). In addition, ES-pH-sensitive-SL demonstrated higher cytotoxic potential as compared to pH-nonresponsive targeted liposomes, by which we can assume that ES-pH-sensitive-SL have improved therapeutic potential even in the condition of restricted residence times at the tumor site.

The plasma profile of ES-SL and ES-pH-sensitive-SL indicated a longer systemic circulation time relative to that of the free drug and suggested greater *in vivo* stability of the formulations following iv administration. The ES-SL and ES-pH-sensitive-SL did not showed significant difference in blood circulation time. The modification of the liposome surface with PEG chains effectively protects liposomes from opsonization, leading to reduction of the RES uptake and prolongation of the circulation time. The concentration of ES-SL-DOX in the blood was found to be higher than that of ES-pH-sensitive-SL-DOX and so to the free DOX (Figure 9). Therefore, it was expected that such liposomes will be preferentially accumulated in solid tumors based on the enhanced permeability and retention (EPR) effect *via* a passive targeting mechanism.

The therapeutic dose of DOX can cause permanent myocardial damage and potentially life-threatening form of cardiomyopathy. The encapsulation of DOX in carrier systems such as liposomes decreases drug associated cardiac complications. The ligand anchored liposomal system effectively delivered drug in higher concentration to the target site along with decreased DOX content in heart. The estrone anchored liposomal formulations ES-SL and ES-pH-sensitive-SL can efficiently deliver DOX into the tumor cells by the receptor-mediated endocytosis and lead to a high concentration of DOX. However, ES-pH-sensitive-SL formulation was more efficient than ES-SL ($P < 0.01$). In the case of heart, liver and kidney tissues the DOX accumulation was significantly reduced. The concentrations of ES-SL and ES-pH-sensitive-SL in liver were lower than those of free DOX at corresponding time points due to RES avoidance and the long circulatory nature of formulations. Thus, the developed formulations efficiently enhance the therapeutic index with minimal toxicity to healthy tissue like heart.

The *in vivo* efficacy of ES-SL-DOX and ES-pH-sensitive-SL-DOX was assayed on breast tumor model (Figure 11). The slight inhibition of tumor growth was observed after injection of free DOX, as reported earlier.[32] The ES-SL showed greater inhibitory effect on tumor growth than that of free DOX. However, ES-pH-sensitive-SL showed maximum inhibition of tumor growth. The high antitumor activity of the targeted liposomal formulations can be attributed to a higher accumulation in cancer cells *via* receptor mediated uptake. However, ES-pH-sensitive-SL was more efficient in tumor suppression when compared with ES-SL, due to accelerated release of DOX from endosomes after receptor mediated endocytosis. These results indicated that inclusion of the pH-sensitivity characteristics increases the efficiency of targeted liposomal system.

The cardiotoxicity of DOX was estimated by measuring serum enzyme activity such as LDH and CPK. The reduced level of LDH and CPK indicated decreased cardiotoxicity of DOX. The ES-pH-sensitive-SL formulation showed further reduced ($p < 0.05$) levels of LDH and CPK, when compared with ES-SL and ($p < 0.01$) when compared with free DOX. These results confirm the significant reduction of cardiotoxic side effects and mortality. Based on the data presented here, it is suggested that ES-modified pH-sensitive-SL could be employed to enhance the intracellular delivery of anticancer agents such as DOX.

5. CONCLUSION

The estrone-modified pH-sensitive liposome system successfully increased therapeutic index of DOX against breast cancer. It enhanced the intracellular uptake of the DOX to receptor positive breast cancer cells and led to improved antitumor activity. The therapeutic improvement of ES-pH-sensitive-SL can be achieved by increased intracellular uptake by ER mediated endocytosis. The ES-modified pH-sensitive-SL could be further employed to enhance the intracellular delivery of anticancer agents such as cytotoxic drugs, proteins, genes or imaging agents, etc. These findings demonstrate a highly

efficient, novel route for targeting anticancer agents to the breast cancer cells.

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