Development and Characterization of pH Sensitive Liposomes Containing Dichloroacetate against Breast Cancer

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Abstract

Background: Breast cancer accounts for 23% of all newly occurring cancers in women worldwide and represents 13.7% of all cancer deaths. The limitation of the available chemotherapeutical agents is largely due to the low accumulation at the tumors relative to their accumulation at normal (healthy) organs due to established multidrug resistance (MDR) which transforms into increased toxicities.

Objective: The objective was to prepare pH sensitive liposomes for targeted delivery of dichloroacetate (DCA) towards breast cancer cells which may reduce the side effects of the formulated anticancer drug, stimulate therapeutic effects, and accomplish targeted anticancer therapy.

Methods: Liposomes were prepared by thin film hydration method. Liposomes were characterized for their size, zeta potential, polydispersity index and percentage drug entrapment efficiency using zetasizer and Sephadex G-50 mini column centrifugation. In vitro cytotoxicity studies were carried out on folate receptor-positive T47D breast carcinoma cells. The in vivo biodistribution studies of developed formulations were performed on Albino mice by intravenous administration.

Results: In vitro cytotoxicity studies revealed that folate appended pH-sensitive formulation was more cytotoxic than conventional liposomes, folate appended conventional liposomes and pH sensitive liposomes. Also, the folate appended pH-sensitive liposomes showed high drug concentration in breast tissue as compared to conventional liposomes, ligand appended conventional liposomes, pH sensitive liposomes and DCA solution.

Conclusion: The findings support that DCA containing folate-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: Breast cancer; Dichloroacetate; Intravenous delivery; pH sensitive liposomes

Graphical Abstract

Dichloroacetate containing pH sensitive liposomes

Anchoring of Ligand specific to the folate receptors over expressed in breast cancer cells

Receptor mediated endocytosis and destabilization of the liposome at endosomal stage of low pH

Drug release intracellularly and killing of cancer cells

Introduction

Cancer is one of the most common and leading cause of death in developed countries. World Health Organization reports that approximately 14 million new cancer cases and 8.2 million cancer related deaths occurred in 2012 [1]. Nowadays, numbers of therapies are used for the treatment of cancer such as chemotherapy, radiation therapy, gene therapy, immunotherapy, surgical therapy and hyperthermia. Among these, chemotherapy is the most commonly used therapy for the treatment of cancer but it has been seen, a number of patients dies only due to the side effects of chemotherapy.

Breast cancer incidence in India is on rise and it becomes number one cancer by pushing cervical cancer to second. Literature shows that during lifetime one in 22 women in India suffer from breast cancer, while in America the figure is definitely more with one in eight being victim of this deadly cancer. One critical point in breast cancer treatment is that only small amount of drug reaches tumor while increasing the concentration of anticancer leads to systemic toxicity and adverse effects [2,3].

Breast cancer is cancer that starts in the tissue of breast. In rare cases breast cancer start in the area of breast. Cancer originating from ducts is known as ductal carcinoma, while cancers that originate from lobules are known as lobular carcinoma [4]. Various
formulation strategies have been exploited to overcome the problem associated with tumor targeting. According to Medicor Cancer Centre, DCA is an analog of acetic acid in which 2 hydrogen atoms of methyl group have been replaced by chlorine atom [5]. The mechanism of action of DCA is initiation of the natural cell suicide system which is repressed in cancerous cells, thus letting them to die on their own. Used with therapy having a poor chance of success to strengthen treatment and to increase success rate DCA preferred when other conventional, scientific proven treatment failed. Normal or healthy cell remain unaffected. Folic acid (FA, folate or vitamin B9), is a vital nutrient which is being used for nucleotide biosynthesis and for the appropriate metabolic maintenance of 1-carbon pathways by all living cells. FA also exhibits high affinity for the folate receptor (FR), a glycosylphosphatidylinositol-linked protein that captures its ligands from the extracellular milieu and carryings them inside the cell via a non-destructive, recycling endosomal pathway [6].

Targeting the folate receptor has shown substantial potential in facilitating the uptake of a variety of drugs when folic acid is conjugated to the drug or delivery vehicle. The folic acid receptors are overexpressed by many types of tumor cells, including ovarian, endometrial, colorectal, breast, lung, renal, neuroendocrine carcinomas, and brain metastases. Folic acid and other targeting molecules have the flexibility to interact with their corresponding cell surface receptor to mediate liposomal uptake via endocytosis, when appended to the terminal end of PEG chains of liposomes.

pH sensitive liposomes are lipid based drug delivery systems that can be swelled due to change in external pH; usually from a neutral or slightly alkaline pH to an acidic pH. They are designed to circumvent delivery of liposome contents to the lysosomes of cells following internalization of the vesicle via the endocytic pathway [7].

In our previous work we have developed folic acid conjugated, docetaxel loaded emulsomes for FR targeting against breast cancer and found profound anticancer efficacy of the developed formulation [2]. In the present work, it was assumed that a pH-sensitive formulation with FR targeting potential could further improve intracellular delivery of DCA to the tumor cells owning intensified levels of FRs, like breast cancer. Therefore, FR targeted pH-sensitive liposomes encapsulating DCA were developed and characterized for the breast cancer treatment.

Materials and Methods

Chemicals

Egg phosphatidylcholine (PC), Sephadex G-50, folic acid, Bismine-PEG (MW=3,350), cholesterylchloroformate, N-hydroxysuccinimide (NHS), Dimethylaminopropyl ethyl carbodiimide (EDC) were purchased from Sigma Aldrich. Dioleoylphosphatidylethanolamine(DOPE) obtained as gift sample from LipoID, Germany. Cholesterol and all other chemicals were of pure analytical grade and used as procured and T47D cell line from Lipoid, Germany. Chloroform and all other chemicals were purchased from NCC Pune.

Preparation of Liposomes

Liposomes were prepared by thin film hydration method [8]. The required quantity of phospholipids in the molar ratios (7:3) each of soy phosphatidylcholine and cholesterol in case of liposomes and the molar ratios (6:3:1) each of soy phosphatidylcholine, cholesterol and DOPE in case of pH sensitive liposomes were dissolved in chloroform in 250ml round bottom flask. The solvent was evaporated in rotary flash evaporator in thermostatically water bath at 37°C (Figure 1). Dried film was hydrated using water containing hydrophilic drug by rotating RBF in opposite direction and resulted in formation of liposome which were then sonicated and liposomes were seen under the motic microscope. Liposomes were characterized for their size, polydispersity index and percentage entrapment efficiency [9]. In case of folic acid appended liposomes, folate-PEG-chol was also added in chloroform.

Synthesis of Folate-PEG-Cholesterol Conjugate

Conjugation of folate-PEG-cholesterol was done in two steps with slight modifications in the process described earlier [10]. Firstly, folic acid was conjugated with Bismine PEG and in second step folate-PEG-NH2 was further conjugated to cholesterylchloroformate.

Preparation of Folate-PEG-NH2:

For synthesis of the folate-PEG-amine, folic acid was dissolved in DMSO (Dimethylsulfoxide) (25mg/ml) along with 1.1M excess of EDC and 1M eq. of NHS and reaction mixture was stirred for 20 min in dark. Then 1M eq. of Bismine PEG was added to the reaction mixture and stirred for 24hrs in darkness at room temperature. The progress of reaction was monitored using pre coated UV active thin layer chromatography plates. The folate-PEG-amine was extracted in mixture of chloroform/water (1:1). Both water and chloroform layers were then concentrated under vacuum and allowed to precipitate by addition of non-solvent, diethyl ether. The conjugate was further characterized by 1H-NMR using DMSO as solvent on Bruker Avance II 400 MHz NMR spectrometer.

Preparation of Folate-PEG-Cholesterol:

To synthesize folate-PEG-cholesterol, a 1.1 molar excess of cholesterylchloroformate was added to folate-PEG-amine in chloroform. The reaction was carried out overnight at room temperature. Progression of the reaction was monitored by the disappearance of free amino group, which was followed by ninhydrin assay. The product, folate-PEG-cholesterol was dried under vacuum, washed twice with ether to remove any residual cholesterylchloroformate and stored at -20°C. The purity of the product was analyzed by silica gel thin-layer chromatography using a solvent system composed of chloroform:methanol (8:2) with trace amount of ammonia. The concentration of folate-PEG-cholesterol was determined by measuring the folate content in the product using UV extinction at 363 nm. Final product was further characterized by 1H-NMR using DMSO as solvent on Bruker Avance II 400 MHz NMR spectrometer.

Optimization of Formulations

Formulations were optimized on the basis of vesicle size, PDI and drug entrapment efficiency. Formulation with high entrapment efficiency and suitable vesicle size were selected as optimum highlighted as bold (Table 1, 2, 3, 4).

Characterisation of liposomes

Morphology: Transmission electron microscopy (TEM FEI, Morgagni 268 D Netherlands) was employed to surface of vesicle imaging for visualization of shape and morphology of the prepared liposomes formulation [9]. A drop of the sample was placed on to a carbon coated copper grid to leave a thin film on the grid. The film was negatively stained with 1% phosphor tungstic acid solution and dried. A drop of the staining solutions was added on to the film and the excess of the solution was drained off. The grid was allowed to
Figure 1: Schematic representation of steps involved in the formation of liposomes.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Ratio (PC: cholesterol)</th>
<th>Hydration Volume</th>
<th>Drug (mg)</th>
<th>Vesicle size (nm)</th>
<th>PDI</th>
<th>% Entrapment Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>6:4</td>
<td>3</td>
<td>2</td>
<td>815.4±7.6</td>
<td>0.505±0.03</td>
<td>15.4±0.9</td>
</tr>
<tr>
<td>F2</td>
<td>8:2</td>
<td>3</td>
<td>2</td>
<td>622.1±5.4</td>
<td>0.128±0.01</td>
<td>25.2±1.1</td>
</tr>
<tr>
<td>F3</td>
<td>7:3</td>
<td>4</td>
<td>2</td>
<td>438.9±6.8</td>
<td>0.333±0.02</td>
<td>42.4±2.1</td>
</tr>
<tr>
<td>F4</td>
<td>7:3</td>
<td>3</td>
<td>1</td>
<td>289.2±4.6</td>
<td>0.347±0.03</td>
<td>20.6±0.9</td>
</tr>
<tr>
<td>F5</td>
<td>7:3</td>
<td>3</td>
<td>2</td>
<td>199.4±5.2</td>
<td>0.291±0.01</td>
<td>48.1±2.6</td>
</tr>
<tr>
<td>F6</td>
<td>7:3</td>
<td>3</td>
<td>3</td>
<td>325.3±5.9</td>
<td>0.383±0.03</td>
<td>31.7±1.8</td>
</tr>
<tr>
<td>F7</td>
<td>7:3</td>
<td>3</td>
<td>4</td>
<td>461.4±5.1</td>
<td>0.347±0.02</td>
<td>37.6±1.9</td>
</tr>
<tr>
<td>F8</td>
<td>7:3</td>
<td>3</td>
<td>5</td>
<td>546.2±4.7</td>
<td>0.283±0.03</td>
<td>32.7±1.4</td>
</tr>
</tbody>
</table>

Table 1: Optimization of conventional liposomes terms of phospholipid ratio, hydration volume and drug. (Mean ± SD; n = 3).
<table>
<thead>
<tr>
<th>Formulation</th>
<th>Ratio (PC: cholesterol: DOPE)</th>
<th>Hydration Volume</th>
<th>Drug (mg)</th>
<th>Vesicle Size (nm)</th>
<th>PDI</th>
<th>% Entrapment Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>5.5:3:1.5</td>
<td>3</td>
<td>2</td>
<td>354.7±4.6</td>
<td>0.241±0.01</td>
<td>17.6±0.9</td>
</tr>
<tr>
<td>S2</td>
<td>5:3:2</td>
<td>3</td>
<td>2</td>
<td>481.2±3.2</td>
<td>0.287±0.02</td>
<td>21.4±1.1</td>
</tr>
<tr>
<td>S3</td>
<td>6.5:3:0.5</td>
<td>3</td>
<td>2</td>
<td>261.7±1.9</td>
<td>0.331±0.03</td>
<td>18.7±0.7</td>
</tr>
<tr>
<td>S4</td>
<td>6:3:1</td>
<td>4</td>
<td>1</td>
<td>509.5±2.8</td>
<td>0.339±0.02</td>
<td>29.5±1.3</td>
</tr>
<tr>
<td>S5</td>
<td>6:3:1</td>
<td>3</td>
<td>2</td>
<td>284.7±1.7</td>
<td>0.336±0.03</td>
<td>42.8±2.4</td>
</tr>
<tr>
<td>S6</td>
<td>6:3:1</td>
<td>3</td>
<td>3</td>
<td>379.4±2.8</td>
<td>0.376±0.02</td>
<td>39.1±1.6</td>
</tr>
<tr>
<td>S7</td>
<td>6:3:1</td>
<td>3</td>
<td>4</td>
<td>373.4±2.1</td>
<td>0.481±0.03</td>
<td>28.6±1.1</td>
</tr>
<tr>
<td>S8</td>
<td>6:3:1</td>
<td>3</td>
<td>5</td>
<td>398.2±2.7</td>
<td>0.325±0.02</td>
<td>31.5±1.7</td>
</tr>
</tbody>
</table>

Table 2: Optimization of pH sensitive liposomes in terms of phospholipid ratio, hydration volume and drug. (Mean ± S.D; n = 3)

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Ratio (PC: cholesterol)</th>
<th>Folate-PEG-Chol</th>
<th>Vesicle Size</th>
<th>PDI</th>
<th>% Entrapment Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>7:3</td>
<td>0.5</td>
<td>230.4±1.2</td>
<td>0.421±0.02</td>
<td>20.8±1.2</td>
</tr>
<tr>
<td>H2</td>
<td>7:3</td>
<td>1.0</td>
<td>242.3±1.6</td>
<td>0.291±0.03</td>
<td>38.6±1.7</td>
</tr>
<tr>
<td>H3</td>
<td>7:3</td>
<td>1.5</td>
<td>248.5±1.8</td>
<td>0.346±0.02</td>
<td>17.7±1.1</td>
</tr>
<tr>
<td>H4</td>
<td>7:3</td>
<td>2.0</td>
<td>236.8±1.1</td>
<td>0.453±0.01</td>
<td>34.8±1.9</td>
</tr>
<tr>
<td>H5</td>
<td>7:3</td>
<td>2.5</td>
<td>278.3±2.1</td>
<td>0.323±0.03</td>
<td>22.3±1.6</td>
</tr>
</tbody>
</table>

Table 3: Optimization of ligand appended conventional liposomes in terms of phospholipid ratio, Folate-PEG-Chol. (Mean ± S.D; n = 3)

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Ratio (PC: cholesterol:DOPE)</th>
<th>Folate-PEG-Chol</th>
<th>Vesicle Size</th>
<th>PDI</th>
<th>% Entrapment Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>6:3:1</td>
<td>0.5</td>
<td>218.4±1.4</td>
<td>0.332±0.02</td>
<td>15.4±0.08</td>
</tr>
<tr>
<td>N2</td>
<td>6:3:1</td>
<td>1.0</td>
<td>323.8±2.3</td>
<td>0.306±0.04</td>
<td>46.5±2.3</td>
</tr>
<tr>
<td>N3</td>
<td>6:3:1</td>
<td>1.5</td>
<td>253.6±1.3</td>
<td>0.421±0.03</td>
<td>32.7±1.7</td>
</tr>
<tr>
<td>N4</td>
<td>6:3:1</td>
<td>2.0</td>
<td>278.7±1.7</td>
<td>0.453±0.02</td>
<td>29.6±1.3</td>
</tr>
<tr>
<td>N5</td>
<td>6:3:1</td>
<td>2.5</td>
<td>302.1±2.1</td>
<td>0.289±0.01</td>
<td>19.3±0.07</td>
</tr>
</tbody>
</table>

Table 4: Optimization of ligand appended pH sensitive liposomes, liposomes in terms of phospholipid ratio and Folate-PEG-Chol. (Mean ± S.D; n = 3).

Volume of hydration (3ml) and drug ratio (2 mg) were kept constant in all preparations.

**Entrapment Efficiency**: The free un-entrapped drug was removed by passing the dispersion through a Sephadex G-50 column [13]. The vesicles were disrupted using 1.0 ml of 0.1% (v/v) Triton X-100 and the liberated drug was estimated spectrophotometrically using a UV- VIS Spectrophotometer in triplicates.

In vitro Drug Release

In vitro drug release profile of entrapped drug from liposomes formulation was studied in PBS pH-7.4 and in PBS pH-5 using dialysis bag. Dialysis membrane was exposed to running water for 12 hours to remove glycerin based contents. The sulfur based contents were removed by treating it with 0.3 % (w/v) sodium sulfide at 80°C for 1 min. Dialysis membrane was washed with hot water at 60°C for 2 min, followed by acidification with 0.2% (v/v) solution of sulfuric acid then rinsed with hot water to remove acid. Treated membrane was kept in alcohol until used for in-vitro drug release studies.
In vitro drug release studies were performed by dialysis bag method using shaking incubator at rotation speed of 100 rpm. Phosphate buffer (pH 7.4) and PBS pH 7.4 were used as dissolution medium. Each dialysis bag (pore size: 12 KD, Sigma Chemical Co., USA) was loaded with liposome formulation equivalent to 800 μg of drug.

Volume and temperature of dissolution medium were 100 ml and 37.0 ± 0.2°C respectively. At predetermined time interval samples (3 ml) were withdrawn, replaced with same volume of fresh media, filtered and assayed for drug content at 218.10 nm against blank using UV-Visible spectrophotometer. Mean results of triplicate measurements and standard deviations were recorded [14].

**In vitro Cell Line Study**

Cytotoxicity Studies (MTT assay)

Cell viability was tested using MTT assay based on the cleavage of yellow tetrazolium salt MTT by metabolically active cells to form an orange formazan dye which was quantified using ELISA (enzyme linked immuno sorbent assay) reader[15,16]. T47D Cells were seeded in 96 well microtitre plates (2 × 104 cells /200 μl growth medium/well) followed by overnight incubation. Supernatants from the wells were aspirated out and different formulations were added.After incubation at 37 ± 1°C for 24h, 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 DMSO. Absorbance at two wavelengths (570 nm for soluble dye and 630 nm for cells) were recorded using ELISA reader. Concentration of samples showing 50% reduction in cell viability i.e. (IC 50 Value) was then calculated.

**In vivo Bio-Distribution Study**

The study protocol for in vivo bio-distribution studies was approved by Institutional Animals Ethical Committee (Ref: ISF/ CP/CSEIAEAC/2012-2013/99). The study was carried out with the guidelines of Council for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Female albino mice (water and food ad libitum) weighing about 20-24 gm were divided in five groups of 9 mice each. Animals were administered with DCA (10mg) solution, conventional, pH sensitive, folate appended conventional and folate appended pH sensitive liposomal formulations through iv route and were sacrificed after 12, 24 and 48 hours. Visceral organs (liver, heart, breast and kidney) of the dissected mice were removed, washed to remove any adhered debris and dried using a tissue paper. The isolated organs were weighed separately, and homogenized in phosphate buffer using tissue homogenizer. The homogenized tissues were deproteinized with 100μl of acetonitrile and kept in dark for 30 min and filtered. The filtrate was then centrifuged at 10000 rpm for 20 minutes. The supernatant was separated and drug content was measured using UV-VIS Spectrophotometer.

The supernatants from successive extracts of an organ from each mouse were pooled and the drug content was determined. The amount of drug in each organ was calculated as percent drug recovered from the respective organ at different time intervals [17].

**Statistical Analysis**

All the experiments were carried out thrice, independently. The data obtained were expressed in terms of ‘mean ± SD’ values. Wherever appropriate, the data were also subjected to unpaired two tailed Student’s t-test. A value of p < 0.05 was considered as significant.

**Results**

Preparation of Folate-PEG-Cholesterol Conjugate

Folate-PEG-Cholesterol was synthesized in two step reaction. Firstly, conjugation of folic acid with Bisamine PEG was conducted by using NHS and EDC as coupling agents. Product was extracted in chloroform layer and precipitated by addition of diethyl ether. When analyzed by gel thin layer chromatography, the folate-PEG-amine was appeared as a single spot with an Rf of 0.82. In second step, folate-PEG-amine was further conjugated to cholesterychlorofomrate as described earlier. Figure 2, shows the 1H-NMR of the final conjugate i.e. folate-PEG-cholesterol! The interpretation revealed the presence of folic acid by the signals in 6.61-8.63 regions. Incorporation of cholesterol was confirmed from the integration value for the protons between 0.82-4.48 regions, which included the signals for the protons of PEG. In comparison with 1H-NMR for folate-PEG-amine and the final NMR, it can be concluded that the conjugation of cholesterol to folate-PEG-amine has been achieved (Figure 2).

**Vesicle Size, Entrapment Efficiency and PDI of Different Optimized Formulations**

The average vesicle size of optimized conventional liposomes, folate appended conventional liposomes, pH sensitive liposomes and folate appended pH sensitive liposomes were found to be 199.4 ± 5.2, 242.3 ± 1.6, 284.7 ± 1.7 and 323.8 ± 2.3 nm respectively. Entrapment efficiency and PDI of optimized conventional liposomes, folate appended conventional liposomes, pH sensitive liposomes, folate appended pH sensitive liposomes were found to be 48.1 ± 2.6, 30.6 ± 1.7, 42.8 ± 2.4 and 46.5 ± 2.3 % and 0.291 ± 0.01, 0.291 ± 0.03, 0.336 ± 0.03 and 0.306 ± 0.04 respectively. The optimized formulations are highlighted as bold (Tables 1, 2, 3, 4).

**Cytotoxicity Study**

In vitro cytotoxicity studies carried out on T47D breast carcinoma cells revealed that folate appended pH-sensitive formulation was more cytotoxic than conventional liposomes, folate appended conventional liposomes, pH sensitive liposomes and drug solution. This may be due to targeted delivery of folate appended pH sensitive liposomes. In vitro cytotoxicity studies carried out on folate receptor positive T47D breast carcinoma cells revealed that folate appended pH-sensitive formulation was more cytotoxic than conventional liposomes, folate appended conventional liposomes and pH sensitive liposomes. This may be due to targeted delivery of folate appended pH sensitive liposomes.
The IC50 value of targeted folate conjugated pH sensitive liposomes was significantly lower than that of conventional liposomes, folate appended conventional liposomes and pH sensitive liposomes in folate receptor positive T47D cells. These results indicate that folate was effective in promoting the internalization of liposomes encapsulating DCA to the folate receptor positive target tumour cells whereas fast release of DCA from endosome by the attack of lysosomal enzymes resulted in the liberation of DCA into the cytosol from where it reaches to the nucleus. This pH dependent mechanism also resulted in higher cytotoxicity. Folate appended conventional liposomes showed little less cytotoxicity then folate appended pH sensitive liposomes. Whereas pH sensitive liposomes showed less cytotoxicity then folate conjugated liposomes (ligand not anchored here) and more cytotoxicity then conventional liposomes because of its pH dependent mechanism (Figure 5).

**In vivo Bio-distribution Studies**

In vivo bio-distribution studies of developed formulations were performed in female albino mice and results are shown in fig 6. In case of conventional liposomes % DCA accumulated in the breast tissue, kidney, liver and heart was found to be 15.2 ± 3.5, 20.6 ± 4.2, 29.7 ± 3.2 and 2.3 ± 0.08 % after 12 hrs. Whereas in case of folate appended conventional liposomes % DCA accumulated in the breast tissue, kidney, liver and heart was found to be 23.66 ± 3.5, 23.2 ± 4.8, 33.66 ± 5.5 and 1.8 ± 0.1 % after 12hr. Similarly pH sensitive liposomes accumulated 29.4 ± 5.8, 17.80 ± 2.8, 19.60 ± 3.6 and 0.9 ± 1.5% of DCA in the breast tissue, kidney, liver and heart respectively whereas folate appended pH sensitive liposomes accumulated 52.66 ± 6.5, 14 ± 2.3, 20.33 ± 3.7 and 1.3 ± 0.09% of DCA in the breast tissue, kidney, liver and heart after 12hr (Figure 6). In case of drug solution, % DCA accumulated in breast tissue, kidney, liver and heart was found to be 10.6 ± 2.9, 7.3 ± 1.5, 19 ± 2.4 and 8.3 ± 2.1 % after 12 hr (data not shown). The results indicate that folate appended pH sensitive liposomes are better in accumulating drug in breast tissue as compared to other organs and other formulations.

**Discussion**

Liposomes represent unique biomaterial, with potential to encapsulate and deliver a variety of therapeutic drugs to target organs. The acidic pH of tumor interstitial fluid, a characteristic of many solid tumors has promoted the development of pH sensitive liposomes with the prospect of selective delivery of anticancer
<table>
<thead>
<tr>
<th>S. No</th>
<th>Formulations</th>
<th>% Drug Release in PBS after 22 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pH 5</td>
</tr>
<tr>
<td>1</td>
<td>Conventional liposomes</td>
<td>50.4 ± 3.2</td>
</tr>
<tr>
<td>2</td>
<td>Folate appended liposomes</td>
<td>48.2 ± 2.4</td>
</tr>
<tr>
<td>3</td>
<td>pH sensitive liposomes</td>
<td>94.4 ± 5.2</td>
</tr>
<tr>
<td>4</td>
<td>Folate appended pH sensitive liposomes</td>
<td>85.8 ± 3.6</td>
</tr>
</tbody>
</table>

Table 5: *In vitro* drug release profile of different formulations at neutral and acidic pH (Mean ± S.D; n = 3)
As folate receptors (FRs) are over expressed in various cancers and folic acid (folate) is a high affinity ligand for the FRs that retains high FR affinity upon derivatization via one of its carbonyl group. Due to its small size and ready availability, folate has become one of the most investigated targeting ligands for tumor-specific drug delivery. Folate has been incorporated into liposomes via conjugation to lipophilic anchors. Folate-conjugated liposomes have been evaluated for targeted delivery of a broad range of therapeutic agents. Therefore it may act as potential target for folate anchored drug delivery.

DCA is very simple molecule; it is an analog of acetic acid in which 2 or 3 hydrogen atoms of methyl group have been replaced by chlorine atom. DCA works by turning on the natural cell suicide mechanism i.e. highest drug release at acidic pH, pH sensitive targeting than pH sensitive liposomes. Due to pH dependent mechanism of release folate appended conventional liposomes showing higher size i.e. 323.8 ± 2.3 nm due to anchoring of ligands and maximum % drug entrainment 46.5 ± 2.3% as compared to other formulations was found to be optimum. Similarly folate appended pH sensitive liposomes (formulation N2) having vesicle size i.e. 323.8 ± 2.3 nm and maximum % drug entrainment 42.8 ± 2.4% as compared to other formulations was found to be optimum. TEM photographs of formulations revealed that pH sensitive liposomes were smooth and spherical in shape, whereas in case of folate appended pH sensitive liposomes anchoring of ligand on liposomes could also be appreciated from its surface as thick boundary (Figure 3 & 4).

The in vitro release profile demonstrated that pH sensitive liposomes of DCA showed 94.4 ± 5.2% release in PBS (pH 5) and 62.7 ± 3.4 % in the PBS (pH 7.4) after 22hr which indicates that formulation might be effective in releasing the drug within acidic tumor environment. However, drug release from folate appended pH sensitive liposomes after 22hr was found to be slightly lesser i.e. 85.8 ± 3.6% in PBS (pH 5) and 54.9 ± 2.1% in PBS (pH 7.4) as compared to pH sensitive liposomes. This may be attributed to hindersance of drug release offered by the ligand anchoring over the liposomes. Similarly release profile of conventional liposome was nearly 50.4 ± 3.2% in PBS (pH 5) and 49.6 ± 2.3% in PBS (pH 7.4) after 22 hr and folate appended conventional liposomes showed slight decrease in release profile i.e. nearby 48.2 ± 2.4 % in PBS (pH 5) and 45.2 ± 1.8 % in PBS (pH 7.4) after 22 hr.

In vitro cytotoxicity studies carried out on T47D breast carcinoma cells revealed that folate appended pH-sensitive formulation was more cytotoxic than conventional liposomes, folate appended conventional liposomes, pH sensitive liposomes and drug solution. This may be due to targeted delivery of folate appended pH sensitive liposomes. The IC50 value of targeted folate conjugate pH sensitive liposomes (IC50-14 mM) was significantly lower than that of conventional liposomes(IC50-44 mM), folate appended conventional liposomes(IC50-26 mM), pH sensitive liposomes (IC50-39 mM) and drug solution(IC50-30 mM) in T47D breast cancer cells. These results indicate that folate was effective in promoting the internalization of liposomes encapsulating DCA into the folate receptor positive breast tumor cells. In folate appended pH-sensitive liposomes, fast release of DCA from endosome by a pH dependent mechanism also be appreciated from its surface as thick boundary (Figure 3 & 4).
that all the formulations showed higher levels of drug in the breast tissue as compared to DCA solution. However, both the conventional and folate appended conventional liposomes showed comparable levels of drug in breast tissues however at the same time these formulations showed comparatively equal or higher drug levels in the liver and kidney. Conversely, pH sensitive liposomes showed significantly higher (p < 0.05) drug levels in breast tissues as compared to conventional and folate appended conventional liposomes. Moreover, folate appended pH sensitive liposomes showed further significantly higher drug levels (p < 0.01) in breast tissues as compared to conventional and folate appended conventional liposomes.

Factualy, the pH of the tumor interstitium rarely declines below pH 6.5, thus rendering it technically difficult to engineer liposomes that become disrupted in such a narrow window of pH. The potential of pH sensitive liposomes lies in their ability to undergo destabilization at the endosomal stage, which presents pH values below 5.0, thereby preventing their degradation at the lysosomal level and promoting the drug’s release into the cytoplasm [7,18].

It is hypothesized, that the liposomes, made up of pH sensitive components, after being endocytosed fuse with the endovacuolar membrane under the action of lowered pH (below 6) and destabilize the endosome, which in turn releases its content directly into the cytoplasm. Other hypotheses claim that the destabilization of pHSLips triggers the destabilization of the endosomal membrane, most likely through pore formation, leading to cytoplasmic delivery of their contents or upon liposome destabilization, the encapsulated molecules diffuse to the cytoplasm through the endosomal membrane [19].
Conclusion

The great advantage of folate conjugated PEG-conventional liposomes and folate conjugated pH sensitive liposomes described in the present study was the simple and fast preparation and the excellent specific folate mediated uptake in folate positive breast cancer cells. Dichloroacetate loaded pH sensitive liposomes were prepared successfully and their drug release was found to be higher at acidic pH as compared to physiological pH which was our primary objective. Such a system for drug delivery provides a platform to formulate anticancer drugs which reduces the side effects of the formulated anticancer drug, promotes therapeutic effects, and achieves targeted therapy. This provides the basis for an efficient targeted delivery of drugs into breast cancer cells preventing side effects induced by the uptake of drug by normal tissues.

Conflicts of Interest

The authors report no conflicts of interest.

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References


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