pH Sensitive Lipid-Polymer Hybrid Nanoparticles Mediated Delivery of Docetaxel: A Viable Approach for Breast Cancer Therapeutic Intervention Development

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Abstract
Present study was planned for the development of pH sensitive lipid polymer hybrid nanoparticles (pHS-LPHNPs) loaded with docetaxel (DTX) for guided and target specific cytosolic-delivery delivery of docetaxel (DTX). pHS-LPHNPs were formulated to entrap DTX by self-assembled nano-precipitation technique and characterised with respect to zeta potential, particle-size, entrapment efficiency, PDI as well as invitro drug release. The cell viability, apoptosis, cellular-uptake, pharmacokinetics, biodistribution in vital organs, % changes in tumour volume and survival of breast cancer bearing animals were used for the evaluation of efficacy of the formulation. In-vitro studies showed increased cytotoxicity at lower IC\textsubscript{50} and better cellular-uptake of pHS-LPHNPs mediated drug by breast cancer cell lines. We saw the better rate of apoptosis of breast cancer cells via Annexin V/Propidium iodide staining. Moreover, in-vivo studies demonstrated improved pharmacokinetics and targetability with minimum drug circulation in deep-seated organs upon delivery of DTX via pHS-LPHNPs in comparison with LPHNPs-DTX and free DTX. We observed sizeable % reduction in tumour-burden with pHS-LPHNPs-DTX than that withLPHNPs-DTX & free DTX. In brief, pHS-LPHNPs mediated delivery of DTX exhibited promising approach for developing therapeutic-interventions against breast-cancer.

1 Introduction
The novel drug delivery systems (NDDS) holds importance in developing therapeutic interventions against cancers, cardiac, and infectious diseases [1, 2]. The novel nanoparticles show advantages over other conventional therapeutic approaches by means of structural rigidity, targeted & controlled release and longer shelf-life [3]. However, systemic administration of polymeric nanoparticles is not recommended due to their swift uptake by the reticuloendothelial system (RES) [4–6]. This rapid uptake of polymeric nanoparticles by RES has been refrained by conjugating the lipids and polymers with polyethylene glycol (PEG) [7–10]. PEG was used to conjugate to a bio-specific ligand that could target and help deliver the entrapped content from the nanoparticles to the preferred site of action [11–15]. The effective delivery at the preferred site and cytosolic delivery of drugs are important for effective treatment [16, 17]. The intracellular uptake might be facilitated via some internal ligands i.e. Folate [16], transferrin [18], lectins [19], Peptides like TAT [17]. The ligand anchored
nanoparticles mediated delivery should to be able to regulate biological functions required under the influence of local stimuli characteristics such as increased temperature or low pH of targeted pathological sites[20]. Therefore, delivery system with an extended circulation time to achieving significant enhanced permeability and retention (EPR) effect. This directed to the accretion of drug loaded nanoparticles at the target sites, and fusogenic properties facilitate fusion of drug delivery system (DDS) with cell membranes, and allow uptake by the target cells and destabilization of endosomes[21]. The controlled cytosolic delivery of bioactives is one of the major hurdles in the drug delivery for developing treatment alternatives against tumors. Therefore our work reports the development of pH sensitive lipid polymer hybrid nanoparticles (pHS-LPHNPs) to deliver drugs into the cytosol directly to increase the drug efficiency by avoiding the endocytic pathway and renders protection of drug from the lysosomal degradation.

pHS-LPHNPs comprise of polymeric core encapsulating hydrophobic drugs, and ac coating of pH sensitive phospholipids[22]. pHS-LPHNPs has greater structural integrity, better drug entrapment, controlled release, excellent serum and storage stability, higher biocompatibility and bioavailability with better tumor targeting capability [23, 24].

Docetaxel (DTX) has been known to inhibits the growth of tumors by various mechanisms such as inhibition of microtubule formation[25], regulating apoptotic pathway[26], inhibiting angiogenesis[27] & cellular signalling pathways[28]. Also, conventional DTX formulation (Taxotere™) raises concerns due to its adverse effects such as neurotoxicity, allergic reactions, reduced uptake by tumors as well as the fluid retention[29, 30].

The issues associated with the delivery of DTX may be overcome by the encapsulation of DTX into pHS-LPHNPs to achieving the selective cytotoxicity in the breast cancer therapy. Thus we decided to explore the comparative delivery potential of DTX encapsulated pH sensitive LPHNPs with non-pH sensitive LPHNPs for developing effective treatment strategies against breast cancer[12].

Our group developed a one step, self-assembled pHS-LPHNPs to ameliorate the encapsulation and cytosolic delivery of hydrophobic drug (DTX) for the effective cancer therapy[10]. In brief, present study shows the evaluation of anti-tumour properties of DTX-loaded pHS-LPHNPs in breast cancer.
cells i.e. MDA-MB-231 & MCF-7. Further, *in vitro* findings were complemented well by the data obtained in an experimentally induced breast cancer animal model. We believe our results surface the targeted and controlled drug delivery potential of pHS-LPHNPs to improve the therapeutic effectiveness of DTX.

2 Materials And Methods
2.1 Reagents
Poly (D,L-lactide-co-glycolide) (PLGA), (50:50) and dioleoyl-phosphoethanolamine (DOPE), Oleic acid and DTX (Sigma-Aldrich, USA), DTX injection (DTX 80 mg/8 ml vial Zydus cadila, India), DSPE-PEG$_{2000}$-$\text{NH}_2$ (Avanti Polar Lipids Inc. Alabama, USA), Cytometric bead array (CBA) kit (BD Biosciences, USA) were procured. Lutrol® F-87 was obtained as sample from BASF, Mumbai, India. All other chemicals and reagents were of analytical grade unless otherwise specified.

2.2 Formulation of pHS-LPHNPs
DTX encapsulated pHS-LPHNPs (pHS-LPHNPs-DTX) were prepared following the method reported by our group[10]. In brief, DOPE and oleic acid (4:1) was dissolved in DMF and heated to 65-70 °C with constant stirring at 500 rpm (Remi, Mumbai, India). The PLGA polymer and DTX were dissolved in DMF at 1 mg/mL. Both lipid and polymer solution were mixed together then mixed with the surfactant solution (0.5%) at the speed of 1 mL/min with moderate stirring and 1 mL of water was added drop wise to the solution. It was stirred for 2 h for the evaporation of the organic solvent. The pHS-LPHNPs were collected, washed and re-suspended in water. To prepare simple LPHNPs, DOPE&Oleic acid was replaced by Egg PC and Cholesterol using the same method as above. The pHS-LPHNPs and LPHNPs encapsulated with coumarin-6 (2 µg/mg polymer) were prepared by replacing DTX with coumarin-6 i.e. pHS-LPHNPs-C6 and LPHNPs-C6 were formulated as described above protocol.

2.3 Formulated pHS-LPHNPs-DTX Characterization
The characterization of prepared nano-formulations were carried out for their size, zeta potential ($\zeta$), Polydispersity index (PI), %drug entrapment (%DE), % drug loading (%DL) and surface morphology. Size and PI of LPHNPs and pHS-LPHNPs were assessed by using Zetasizer (Malvern Panalytical, UK) [12]. The surface characteristics of the pHS-LPHNPs were evaluated by Field Emission Scanning
Electron Microscopy (FE-SEM) (SU8010, Hitachi, Switzerland)[11].

The height and diameter of the pH-S-LPHNPs was done by atomic force microscopy (AFM, Multimode-8 HR, Bruker USA). In this method, pH-S-LPHNPs (10 µl) were carefully put on a silicon wafer. De-ionized water was used to remove the excess stuff from the surface. Then the sample was dried to create a thin film and scanned by placing under the lens of AFM, and three dimensional structures were observed [31].

Percent drug entrapment (%DE) was calculated by using lysis technique. In brief, lyophilized pH-S-LPHNPs in predetermined quantity of were mixed with 5 mL solution of ACN: H$_2$O (6:4) and sonicated for 3 min. The solution was filtered and further diluted with ACN: H$_2$O(6:4) and charged onto HPLC system (e2695, Waters, Milford, USA)[32]. Percent drug entrapment (%DE) and percent drug loading (%DL) were estimated by using the following equations[11]

\[
% \text{DE} = \frac{\text{Amount of drug in pH-S-LPHNPs}}{\text{Total amount of drug added}} \times 100
\]

\[
% \text{DL} = \frac{\text{Amount of drug entrapped in pH-S-LPHNPs}}{\text{Total weight of pH-S-LPHNPs}} \times 100
\]

2.4 *In vitro* DTX release from pH-S-LPHNPs

To evaluate the pH sensitivity of prepared pH-S-LPHNPs, the *in vitro* DTX release in two different buffered saline of pH 7.4 & 5.5 was investigated by the dialysis tubing. In Brief, 2 mL of pH-S-LPHNPs-DTX formulation was kept in a dialysis bag (MWCO 10 kDa) that was stirred at 100 rpm in the dialysis solution at 37 ± 1 °C [10]. At pre-determined time schedule, 0.5 mL of medium was taken out and same quantity of fresh medium was added at each withdrawal. These samples were assayed to estimate the drug content by HPLC at 230 nm[33].

2.5 pH induced aggregation of pH-S-LPHNPs

Aggregation of pH-S-LPHNPs due to low pH was observed by the estimation of particle size. About 50 µL of pH-S-LPHNPs was mixed to 5 mL of 7.4 and 5.5 pH bufferseach and incubated at 37 ± 1 °C. Samples were taken and particle size was measured at predetermined time intervals.

2.6 *In vitro* Studies
2.6.1 Cell culture
Human breast cancer cell lines i.e. MDA-MB-231 & MCF-7 cells were cultured and maintained with minor modifications in the protocols reported elsewhere [10, 34]. In brief, both the cell lines were cultured in 5% CO₂ atmosphere at 37 °C in Dulbecco’s modified eagle media (DMEM). The cells were harvested with 0.25% trypsin and 1% EDTA solution upon reaching 80% confluency and diluted as per the requirement.

2.6.2 Cell viability studies
About 1 × 10⁴ cells/well in 96 wells culture plates were seeded (Tarsons Products Pvt Ltd, Kolkata, India) [12, 34]. After 24 hr, culture media was replaced with the medium containing the DTX, LPHNPs-DTX, pHS-LPHNPs-DTX to the respective wells at concentration equivalent to 0.05, 0.1, 1, 10, and 20 µg/mL of free DTX and incubated for an additional 24, 48 and 72 hr. The wells were flushed with physiological PBS and then 100 µL of MTT solution (1.25 mg/mL in PBS) was added to each well followed by re-incubation for 4 hr to allow the formazan crystals formation. About 200 µl of DMSO was added to each well to solubilize the formazan crystals. The untreated cells were taken as control. The absorbance (Abs) of obtained solution was recorded at 570 nm by 96 well plate reader (BioTek instruments, USA). The cell viability was calculated by using the following formula[35]. CalcuSyn software was used to calculate IC₅₀ values via median-effect plot.

\[
\text{Cell viability (\%) = } \frac{\text{Abs of sample} - \text{Abs of Blank}}{\text{Abs of control} - \text{Abs of Blank}} \times 100
\]

2.6.3 Qualitative and quantitative cell uptake studies
The in-vitro studies for cell uptake were performed as described elsewhere[12]. The qualitative cell uptake of pHS-LPHNPs with MDA-MB-231 & MCF-7 cells was estimated by using confocal microscopy (CLSM). The cells were inoculated on coverslip (Tarsons Products Pvt Ltd, Kolkata, India) about 5 × 10⁴ cells/well placed in 6-well plate and incubated for 24 hr at 37 °C. Cells were incubated with pHS-LPHNPs (1 µg/mL equivalent to C6) for 2 hr at 37 ± 1 °C followed by washing with PBS (three times) to
eliminate the extracellular particles. Cells were fixed with 4% PFA for 20 min at 20 °C. The coverslips were fixed on slides and observed with CLSM (eclipse Ti, Nikon).

The quantitative evaluation of cell uptake was conducted by HPLC analysis of DTX [33]. Both the cells were inoculated (5 × 10^4 cells/well) in 24 wells plates. These were incubated overnight to allow the cells to adhere. Growth media was replaced with media containing pH5-LPHNPs-DTX (equivalent to 10, 20, 30, 40 µg of DTX) and again incubated for 2hr to see the effect of concentration on cellular uptake. The washing of cells (3 times) were carried out with PBS and lysed with 0.1% Triton™ X-100 after incubation. The internalized DTX was extracted with ACN: H₂O (6:4) and cell lysate was centrifuged for 15 min at 25,000 rpm. The supernatant was analysed by HPLC.

2.6.4 Detection of apoptosis by annexin V-FITC/propidium iodide assay

Apoptosis assays were performed as described previously [12, 36]. In brief, 5 × 10^4 MDA-MB-231 cells/well cells were seeded in six-well culture plates. The cells were distributed in three groups (three wells per group): (i) free DTX (10 µg/ml) (ii) LPHNPs-DTX (10 µg/ml) &pHS-LPHNPs-DTX (10 µg/ml). The apoptosis assays were performed to detect apoptosis by the FITC Annexin V Apoptosis Detection Kit (BD Biosciences, USA) using flow cytometer (BD FACSCanto II, BD Biosciences, USA) according to the manufacturer’s protocol.

2.7 In-vivo study

All the experimental protocols were approved by Institutional Animal Ethical Committee of Jilin University, Changchun, China. The animal care and experimental protocol were followed as per the guidelines of the animal ethics committee. The pharmacokinetic, bio-distribution, and anti-tumor studies were performed as reported earlier[12, 37]. 25-30gBALB/c female mice were acclimatized at 25 ± 1 °C and 45 – 55% RH with natural day/night conditions with ad libitum food and water for 7 days before the commencement of experiments. In order to develop a xenograft model, approximately 4 × 10^5 MDA-MB-231 cells with 50% matrigel in 50 µL of culture medium injected subcutaneously into the mammary fat pad of female mice[38].
Tumor volume was measured by the following equation.

\[ V = \frac{1}{2} (\text{Length} \times \text{Width}^2) \]

Where length and width of the tumour was measured by a Vernier calliper

### 2.7.1 Pharmacokinetics study

Animals were kept in 3 groups consisting 6 animals each. The group I, II and III were injected asingle dose of free DTX, LPHNP-DTX &pHS-LPHNP-DTX (equivalent to 10 mg/kg of DTX) through intravenous route. 200µl of blood wasdrawn through the puncture of retro-orbital sinus at regular time intervals with the partial anaesthesia and stored at -20 °C until analysed. 300 µl acetonitrile (ACN) was added to 100 µl of plasma to remove proteins, then centrifuged to obtainsupernatants and analysed for DTX[33]. The kinetica software (Thermo-scientific, USA) was used to study pharmacokinetic parameters such as peak plasma concentration (\(C_{\text{max}}\)), half-life (\(t_{1/2}\)), area under the curve (AUC), mean residence time(MRT), and time to achieve maximum plasma concentration(\(T_{\text{max}}\)).

### 2.7.2 Bio-distribution study

Bio-distribution of drug was estimated on xenograft BALB/c female mouse model. Animals were divided in 3 groups with 6 animals each. After 24 hr of single dose administration(DTX, LPHNPs-DTX and pHSL-PHNPs-DTX), animals were euthanized and highly perfused organs like liver, heart, kidney, spleen, lungs and tumor were removed and weighed. 20% tissue homogenate was prepared in normal saline and stored at -20 °C. 300 µL of ACN was added to 100 µL of tissue homogenate to remove the proteins followed by centrifugation, filtrationand analysis [33], and the distribution pattern was evaluated in highly perfused organs.

### 2.7.3 In Vivo anti-tumour activity

The anti-tumor activity of pHSL-PHNPs-DTX wereevaluated in experimentally induced breast cancer mouse model[12]. Animals were randomly distributed into 4 groupsconsisting 6 animals each. Group 1, 2, &3 received DTX, LPHNPs-DTX and pHSL-PHNPs-DTX, respectively. The 4th group received normal saline and served as an experimental control. Formulations (equivalent to 6 mg/kg DTX) were administeredthrough lateral tail vein twice a week for 5 weeks, and tumor size wasmeasured after 5
weeks of treatment.
The blood samples were withdrawn via the retro-orbital sinus of animals and analysed for IL-6, IL-1β, MMP1, TNF-α & COX2 following manufacturer’s instructions [39, 40]. The survival rate was calculated with the same groups for 90 days.

3 Results And Discussion
3.1 Preparation and characterization of pHSLPHNPs formulations
DTX encapsulated pHSLPHNPs were prepared using one step, self-assembled nano precipitation techniques[41] (Schematic).
The DTX were encapsulated in the developed pHSLPHNPs in PLGA polymeric core [10, 24]. This core was encircled by crust of semi-polar biocompatible phospholipids along with DOPE and DSPE-PEG layer[41]. The average particle size of LPHNPs-DTX and pHSLPHNPs-DTX was estimated 126.36 ± 4.67 nm and 151.31 ± 6.19, respectively. PI (< 0.18) submits the uniform distribution of pHSLPHNPs-DTX, and zeta potential of pHSLPHNPs-DTX formulation was calculated to be -12.31 ± 0.95 (Table 1).

The atomic force microscopy (AFM) and field emission scanning electron microscopy (FE-SEM) observations of these formulations confirm the spherical shape and nanometric size of formulated nanocarriers (Fig. 1). However, minor difference was seen in particle size when analysed by zetasizer. Percent drug entrapment & drug loading for DTX encapsulated pHSLPHNPs were calculated to be 58.9 ± 2.17% and 10.36 ± 0.43% (w/w), respectively. DTX loading and entrapment efficiency of LPHNPs were calculated 69.1 ± 2.78% & 12.16 ± 0.47% respectively (Table 1).

3.2 In-vitro drug release
In-vitro drug release of pHSLPHNPs-DTX & LPHNPs-DTX was carried out with two different pH buffers (pH 7.4 & 5.5). As expected, DTX % release from pHSLPHNPs was seen greater (42%) at low pH (5.5) within 12 hr whereas only 20% at pH (7.4). This release was trailed by slow and sustained for next 108 hrs. The drug release pattern from LPHNPs-DTX was nearly same but showed minor difference in the cumulative percent of drug release
at both pHs. Fast DTX release from pHS-LPHNPs in acetate buffer (pH 5.5) was observed that may be due to the rapid destabilisation of lipid adsorbed at the outer surface of the PLGA core of pHS-LPHNPs and release of entrapped drug from the polymeric core. The release of DTX from pHS-LPHNP-DTX was significantly (p < 0.05) higher as compared to LPHNPs-DTX upto 120th hr (Fig. 2). These findings are in accordance to the previous studies [10] and validate our findings.

3.3 pH Induced pHS-LPHNPs aggregation

The aggregation of the prepared pHS-LPHNPs-DTX was assessed by the changes seen in particle size and charge due to changes observed in pH. The particle size of pHS-LPHNPs-DTX was seen increased considerably at low pH (Fig. 3) demonstrating particle accumulation & fusion of the outer lipid coating of polymeric core. The aggregation of pHS-LPHNPs-DTX at pH 5.5 was seen (Fig. 3). A gradual increase in the size of pHS-LPHNPs-DTX was seen upon lowering down the pH. However, size of vesicle might not be reduced by increasing the pH again to 7.4. Size of pHS-LPHNPs-DTX increased quickly upon incubation for 30 min at pH 5.5, but further incubation showed a steady growth in vesicle size. The LPHNPs-DTX formulation did not show any assemblage or increase in size of vesicle.

3.4 In vitro cell culture

3.4.1 Cell viability test

Cell viability test of pHS-LPHNPs-DTX was carried out with MDA-MB-231 and MCF-7 cells in concentration & time dependent manner (0.05, 0.1, 1, 10 and 20 µg/ml of DTX; 24, 48, 72 hr respectively) (Fig. 4). pHS-LPHNPs-DTX formulation exhibits greater effect on cytotoxicity in a time-dependent manner upto 48 hr (Fig. 4). The greater cytotoxicity was seen by pHS-LPHNPs-DTX through the fusogenic lipid mediated cytosolic delivery of DTX. The cytotoxic response of DTX encapsulated pHS-LPHNPs leads to proportionate increase with arise in the concentration of drug in the following order: DTX < LPHNPs-DTX < pHS-LPHNPs-DTX.

IC$_{50}$ of free DTX was found 7.8-11.45 µg/mL & 7.84-10.81 µg/mL after 24 hr with MCF-7 and MDA-MB-231 respectively. LPHNPs-DTX and pHS-LPHNPs-DTX displayed a sharp drop in % cell viability as compared to the free DTX (Fig. 4A, B). pHS-LPHNPs-DTX showed significantly higher (p < 0.001) anti-tumour activity than that seen with free DTX and LPHNPs-DTX. Obtained results with % cell viability were in consonance with cell uptake studies showing better uptake of pHS-LPHNPs-C6 by MCF-7 & MDA-MB-231 cell lines. Lower concentration of DTX delivered via pHS-LPHNPs showed better anti-tumour activity in MCF-7 & MDA-MB-231 cell lines than free
DTX&LPHNPs-DTX (Fig. 4).

### 3.4.2 Qualitative and Quantitative assessment of cell uptake

The comparative analysis of intra-cellular uptake of coumarin-6 encapsulated LPHNPs (LPHNP-C6) and pH sensitive LPHNPs (pHS-LPHNPs-C6) was done. The correlation of cell uptake with cytotoxic effect was calculated in MCF-7 & MDA-MB-231 cells. LPHNPs-C6 and pHS-LPHNPs-C6 incubated with both the cells for 2hr for qualitatively assess the rapid internalisation (Fig. 5). The fluorescent signals emitted by LPHNPs-C6 & pHS-LPHNPs-C6 formulations were quantified more than that seen with free C6 formulation (Fig. 5). The signal intensity emitted from the pH Sensitive LPHNPs (pHS-LPHNP-C6) was quantified higher than LPHNPs (LPHNP-C6) (Fig. 5), and results were attested by the quantification of DTX uptake through HPLC method. The increased cell uptake of pH sensitive LPHNPs is accredited with the cytosolic drug delivery [11, 22, 41].

Our quantification results of cell uptake obtained with various concentrations are well supported by the qualitative observations of confocal laser scanning microscopy (CLSM). The cellular uptake results showed that drug uptake was not significantly ($p > 0.05$) proportionate to the concentration, and the greater uptake was seen at 40 µg/ml (Fig. 6A, B). Our results showing the internalisation of pHS-LPHNPs formulations were discovered time-dependent that leads to significant rise ($p < 0.001$) in DTX uptake with change of the incubation time from 1–2 hr [12] (Fig. 6A, left panel 1 & 2; Fig. 6B, Right panel 1 & 2). However, further increase in incubation time from 2–3 hr did not show any significant effect ($p > 0.05$) on cellular uptake (Fig. 6A & B; Left panel 3 and right panel 3). Thus, based on our results, 2 hr incubation time was optimized and chose for subsequent experiments.

The efficiency of cellular uptake of pHS-LPHNPs-DTX was found highest (45–48%) as compared to LPHNPs-DTX (37–39%) whereas the least uptake was estimated with free DTX. These findings suggest that the greater cellular uptake with pHS-LPHNPs-DTX was attributed to the fusion with endosomal membrane and outflow into the cytoplasm from the endosome [12].

### 3.4.3 Detection of apoptosis by annexin V-FITC/propidium iodide (PI) assay

Annexin V-FITC/PI apoptosis assay was conducted to quantify apoptosis at different time points. Q4 quadrant in the figure (Annexin V-positive, PI-negative) shows the percentage of early apoptotic cells, while the cells in Q2 quadrant (Annexin V-positive, PI-positive) are in late apoptosis. pHS-LPHNPs-DTX is shown to induce increased
fraction of early and late apoptosis as compared to LPHNPs-DTX & free DTX (Fig. 7). Treatment of pHS-LPHNPs loaded DTX to MDA-MB-231 cells showed 18.10% cells in the early apoptosis stage as compared to LPHNPs-DTX (10.1%) & free DTX (3.20%) and 22.3% of cells were in late apoptosis as compared to LPHNPs-DTX (16.1%) free DTX (6.9%). Total injured cells (including early & late apoptosis and necrotic cells) with pHS-LPHNPs-DTX was found to be higher (93%) than that seen with LPHNPs-DTX (78%) & free DTX (49%)(Fig. 7). These findings corroborate the results obtained with the quantitative uptake assays, and suggest the better anticancer effect of pHS-LPHNPs-DTX formulation [36]. It happens due to the induction of greater rate of apoptosis of cancer cells

3.4.4 \textit{In vivo} pharmacokinetics and bio-distribution study

The pharmacokinetic studies were carried out to assess the effect of polymers and lipids on the circulation time of different LPHNP formulations. The release of DTX in the blood was assessed (Fig. 8A) and pharmacokinetic parameters were assessed upon administration of single dose in the experimentally-induced breast tumour mice model (Table 2). Our findings confirm the increased circulation residence, improved half-life of DTX when loaded and delivered via pHS-LPHNPs and compared with free DTX formulations. The administered free DTX formulations were found in higher concentration in the circulation up-to 2 hr following are ductin in drug concentration after 6 hr. Furthermore, LPHNPs-DTX and pHS-LPHNPs-DTX formulations when administered through intravenous route showed low initial concentrations in blood, and then showed greater concentration of drug after 6 hr of administration[11]. The decrease in the levels of DTX was seen from 6 to 72 hr. pHS-LPHNP formulations showed detectable serum DTX at the end of 72nd hr.

| Pharmacokinetic parameters | DTX | LPHNP-DTX | pHS-LPHNP-DTX |
|----------------------------|-----|-----------|---------------|
| $C_{\text{max}}$ (µg/mL)   | 15.98 | 7.86     | 8.76         |
| AUC$_{\text{total}}$ (µg/mL*h) | 38.62 | 155.49   | 222.89       |
| $T_{1/2}$ (h)               | 1.76  | 13.42    | 16.03        |
| MRT (h)                     | 3.24  | 21.54    | 25.33        |

The formulations of LPHNP showed 7–8 times higher mean residence time (MRT) of DTX than free DTX. Seven fold MRT was observed with LPHNP-DTX formulations and 8 times with pHS-LPHNPs-DTX formulations (Table 2). Likewise, AUC$_{\text{total}}$ and half-life of LPHNPs-DTX was assessed higher than the free DTX (Table 2). Our results clearly showed the prolonged circulation time of pHS-LPHNP formulations and therefore suggesting the extended retention of DTX in the blood stream (Table 2). Consistent to our previous findings, results of this study advocate
for the sustained and extended release behaviour of DTX when encapsulated into LPHNPs & pH5-LPHNPs [11]. LPHNPs and pH5-LPHNPs showed greater plasma levels of DTX due to the double encirclement effect [19]. Therefore, these pharmacokinetic results suggest potential of pH5-LPHNPs for the effective and efficient delivery of DTX for developing treatment strategies against breast cancer. We believe these bio-macromolecular delivery systems show extended periods of drug retention and improved bioavailability in the experimental animal modes of breast cancer.

DTX was significantly \( (p < 0.001) \) quantified in the tumour sites when experimental animals were treated with pH5-LPHNPs-DTX and compared with LPHNPs-DTX and free DTX. Our findings suggest that freeDTX had distributed to the liver, kidney, spleen, lungs, heart and inadequate quantity of drug reaches to the tumour sites. A sizeable quantity of DTX was seen in liver & spleen in addition to the tumour sites when administered through LPHNPs (Fig. 8B) [12].

3.5 Anti-tumor efficiency of pH5- LPHNPs-DTX

The anti-tumor efficacy of prepared pH5-LPHNPs-DTX formulations was assessed in the experimental xenograft model (Fig. 8A). The treatment of DTX encapsulated pH5-LPHNPs showed significantly reduced \( (p < 0.001) \) in tumour dimensions than that seen with free DTX \( (79.8\%) \) & LPHNPs-DTX \( (36.91\%) \) post 3 weeks of breast cancer drug therapy (Fig. 8B). The residual tumour burden was measured for 3 weeks \( (36.91\%, 21.51\% \& 151.6\%) \) for LPHNPs-DTX, pH5-LPHNPs-DTX and normal saline (positive control), respectively (Fig. 8C). pH5-LPHNPs-DTX formulation showed improved \textit{in vivo} anti-tumour efficiency associated with the greater \textit{in vitro} cell uptake and cytotoxic effect than free DTX and LPHNPs-DTX formulations.

The inflammatory cytokines such as IL-1\( \beta \), IL-6, MMP\( _1 \), TNF-\( \alpha \), & COX-2 are commonly seen markers playing the instrumental role in pathology of breast cancer. MMP\( _1 \) is an enzyme involved in the breakdown of interstitial collagens and found overly expressed during breast cancer pathogenesis [42–44]. Thus, we estimated the MMP1 in serum after therapeutic regimen (Fig. 8D). The higher levels of MMP1 were seen in xenograft model treated with normal saline. However, a sizeable reduction of various inflammatory markers (IL-6, IL-1\( \beta \), MMP\( _1 \), TNF-\( \alpha \), & COX-\( _2 \)) was detected in the serum of breast cancer bearing experimental animals upon treated with pH5-LPHNPS-DTX. The reduction of inflammation was seen closer to the animals from experimental control (without tumor). Our results show that administration of DTX when delivered via pH5-LPHNPs present a better therapeutic
approach than the free DTX for subsiding inflammation induced by the breast cancer pathogenesis (Fig. 8E).

The improved survival of animals treated with these nanoformulations mediated delivery of DTX suggest the beneficial impact shown by DTX throughout 90 days of therapeutic regimen. The repeated administration of pHS-LPHNPs-DTX exhibited lesser mortality (33.33%) as compared to the greater (66.66%) mortality assessed in experimental animals upon treated with free DTX. The lesser mortality arbitrated by pHS-LPHNPs-DTX was shown by the Kaplan – Meier survival analysis for 90 days of tumor development and therapeutic regime of DTX(Fig. 8B).

4 Conclusion
Our results suggest the importance of novel drug delivery vehicles, pHS-LPHNPs for the effective and efficient delivery of DTX[45–48]. The combination of two pathways, 1) pH sensitivity of formulation leads to fast internalization of pHS-LPHNPs by the means of endocytosis and greater accumulation of pHS-LPHNPs-DTX at tumor location with minimum circulation in other organs, and 2) Cytotoxic effect of DTX on cancer cells by affecting the apoptotic pathway[26]. In conclusion, our formulations based on novel approach proved effective for the treatment of breast cancer and open new avenues for developing effective therapeutic interventions against breast cancer.

5 Future Perspectives
Our results show that pH sensitive LPHNPs (pHS-LPHNPs-DTX) formulations are more effective than LPHNP-DTX with respect to cytosolic delivery, reduced cytokine levels and improved bioavailability. Briefly, improved pharmacodynamic & pharmacokinetics and efficiency of pHS-LPHNPs-DTX lead towards better functionality and upgraded strategy for future nanomedicine in which the synergistic effect of formulation, sustained drug release, and specific cytosolic distribution of drug(s) into the cancerous cells should be taken into consideration. The amenable results obtained with pH sensitive nano-carriers suggest a path towards the solution for breast cancer, and warrants further research to explore encapsulation of DTX for breast cancer therapeutics.

Declarations
Ethical approval and consent to participate
Animal ethical approval was sought and mentioned in materials and methods

Consent for publication
Sought

Availability of data and materials
All data is presented in the manuscript

**Competing interests**
The authors declare that they have no competing interests

**Conflicts of Interest**
Authors declare no conflict of interest

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Figures
Figure 1
A) The FE-SEM study of pHS-LPHNPs-DTX; B) Atomic force Microscopy (AFM) of pHS-LPHNPs-DTX

Figure 2
A) The FE-SEM study of pHS-LPHNPs-DTX; B) Atomic force Microscopy (AFM) of pHS-LPHNPs-DTX
Estimation of in vitro drug release from pH5-LPHNPs-DTX & LPHNPs-DTX at different pHs
Figure 4

Estimation of in vitro drug release from pH-S-LPHNPs-DTX & LPHNPs-DTX at different pHs
Mean particle size of pHS-LPHNPs (a) The mean particle size of the pHS-LPHNPs-DTX after 30 min at various pHs, and (b) Size of pHS-LPHNPs-DTX at various time interval in pH 5.5 buffer.
The in-vitro cytotoxicity assay was carried out with MCF-7 cells (A, B). % cell viability was calculated with free DTX, LPHNPs-DTX and pHs-LPHNPs-DTX formulations after 24, 48 and 72 hr. IC50 values were calculated to assess the drug sensitivity by using CalcuSyn software.
The in-vitro cytotoxicity assay was carried out with MCF-7 cells (A, B). % cell viability was calculated with free DTX, LPHNPs-DTX and pHs-LPHNPs-DTX formulations after 24, 48 and 72 hr. IC50 values were calculated to assess the drug sensitivity by using CalcuSyn software.
The qualitative cell uptake study was carried out by using CLSM.
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The quantitative cell uptake study was carried out by the cell lysis method A) MDA-MB-231 cells treated with free DTX, LPHNPs-DTX and pHS-LPHNPs-DTX formulations for 1, 2 & 3 hr with varying concentrations of DTX, B) MCF-7 cells incubated with free DTX, LPHNP-DTX, pHS-LPHNPs-DTX formulations.
The quantitative cell uptake study was carried out by the cell lysis method A) MDA-MB-231 cells treated with free DTX, LPHNPs-DTX and pH5-LPHNPs-DTX formulations for 1, 2 & 3 hr with varying concentrations of DTX, B) MCF-7 cells incubated with free DTX, LPHNP-DTX, pH5-LPHNPs-DTX formulations.
Determination of apoptosis induced by the using annexin V kit through A) flow cytometry, B)pHS-LPHNPs loaded DTX showed greater rate of apoptosis as compared to DTX loaded LPHNPs and free DTX.
Figure 14

Determination of apoptosis induced by the using annexin V kit through A) flow cytometry, B)pHS-LPHNPs loaded DTX showed greater rate of apoptosis as compared to DTX loaded LPHNPs and free DTX
The serum assessment of pharmacokinetics, biodistribution, anti-tumor activity, inflammatory cytokines and animal survival with pHS-LPHNPs-DTX (A) DTX plasma level after one-time intravenous administration of DTX (B) Bio-distribution of DTX after 24 hr (C) Estimation of in vivo anti-tumour activity of pHS-LPHNPs-DTX in xenograft model. Reduction in % tumour burden was estimated post 3 weeks therapeutic regimen, (D) detection of the reduction in the serum levels of pro-inflammatory cytokines after drug therapy, and (E) The Kaplan-Meier survival curve for 90-days therapy.
Figure 16

The serum assessment of pharmacokinetics, biodistribution, anti-tumor activity, inflammatory cytokines and animal survival with pHS-LPHNPs-DTX (A) DTX plasma level after one-time intravenous administration of DTX (B) Bio-distribution of DTX after 24 hr (C) Estimation of in vivo anti-tumour activity of pHS-LPHNPs-DTX in xenograft model. Reduction in % tumour burden was estimated post 3 weeks therapeutic regimen, (D)detection of the reduction in the serum levels of pro-inflammatory cytokines after drug therapy, and (E) The Kaplan-Meier survival curve for 90-days therapy.
Figure 17

Schematic for the development of DTX loaded pH Sensitive lipid polymer hybrid nanoparticles (pHS-LPHNPs-DTX)
Schematic for the development of DTX loaded pH Sensitive lipid polymer hybrid nanoparticles (pHS-LPHNPs-DTX)
