Design of Targeted and Release Controlled Liposome for Paclitaxel and Doxorubicin Combination in Breast Cancer Therapy

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Abstract: Paclitaxel and doxorubicin are commonly used in chemotherapy of breast cancer. Now we have developed a kind of nanocarrier liposome that can deliver the combination of Dox and PTX to alleviate the pain brought by side effects and decrease the resistance. In this paper, we expected the result data of the drug loading capacity and the drug loading efficiency, the release of PTX and Dox in different pH environment, and the absorption of drugs which is estimated by the amount of free drug remains in the cells. The combination of Dox and PTX in liposome was modified with folic acid for tumor targeting, and achieved pH responsive drug release in tumor cell by introduction of N-(4-carboxybenzyl)-N, N-dimethyl-2,3-bis (oleoyloxy) propan-1-aminium (DOBAQ), a kind of pH sensitive lipid. The double layers were loaded with hydrophobic drug PTX, and the hydrophilic drug DOX is loaded in the aqueous core of the vesicle. We expected the results to reduce side effects and improve specificity when treating the cancer.

1 INTRODUCTION

Breast cancer is the disease in which breast epithelial cells proliferate out of control under the action of a variety of carcinogens. The early stage of the disease often manifests as breast lumps, nipple discharge, axillary lymphadenopathy, and other symptoms (Yin, 2010). In the late stage, cancer cells may metastasize to a distance, and multiple organ diseases may appear, which directly threaten the life of the patient. Breast cancer is often called the "pink killer", and its incidence ranks first among female malignant tumors. Male breast cancer is relatively rare. With the improvement of medical treatment, breast cancer has become one of the solid tumors with the best curative effect. Risk factors for breast cancer include genetic factors, hormonal changes, mental and psychological factors, and history of past breast diseases. According to the latest data from the International Agency for Research on Cancer (IARC) in 2018, the incidence of breast cancer in female cancers worldwide is 24.2%, ranking first among female cancers, of which 52.9% occur in developing countries (Feng, 2021).

Breast cancer treatment is personalized. All patients need to be formulated by authoritative experts based on their own conditions. The treatment plan depends on many factors, including tumor subtypes, patient's age, general health, menopause and eating habits, stage of the tumor, hormone receptor status (ER, PR) and HER2 status, genetic information (such as BRCA1 or BRCA2), and genetic testing results, such as full genetic testing and breast cancer 21 gene testing (Oncotype DX™) (Reddy, 2011). At present, the conventional methods of treatment of breast cancer mainly include surgery, radiotherapy, chemotherapy, hormone therapy, targeted therapy, immunotherapy, etc. Radiation therapy, chemotherapy, targeted therapy and/or hormone therapy can be used to assist the treatment (Bodei, 2007).

1.1 Surgery

For ductal carcinoma in situ and early invasive breast cancer, doctors usually recommend surgery to remove the tumor (Trayes, 2021). Most patients with invasive breast cancer will undergo sentinel lymph node biopsy or axillary lymph node dissection. Most important thing in the treatment of early- stage breast cancer is to reduce the risk of recurrence by removing

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all remaining cancer cells. These cancer cells are undetectable, but they can cause cancer to recur because they grow over time.

1.2 Radiation Therapy

Radiation therapy uses high-energy X-rays or proton rays to destroy cancer cells. Radiation therapy helps reduce the risk of breast recurrence. Through surgery and radiotherapy, within 10 years of treatment, the recurrence rate of breast cancer is now less than 5 % (McGale, 2014). In the past, traditional radiotherapy increased the long-term risk of heart disease in women with left breast cancer. Now, proton therapy can protect the heart from radiation damage. Therefore, more breast cancer patients can reasonably obtain a longer survival period and better quality of life through modern medical methods.

1.3 Medication

Systemic therapy using medication can kill cancer cells comprehensively. Types of systemic therapy for breast cancer include chemotherapy, hormone therapy, targeted therapy, and immunotherapy (Bodei, 2007).

Chemotherapy began in the 1940s and 1950s, and its application has greatly improved the efficiency of anti-tumor. Although surgery can remove tumor tissue, it cannot be completely removing all cancer cells, especially for circulating cancer cells in the blood. At this time, chemotherapy is needed to kill the remaining cancer cells and reduce the risk of recurrence and distant metastasis. Therefore, chemotherapy is of great significance to tumor treatment (Hassan, 2010). In the classification and treatment of breast cancer today, chemotherapy also plays a very important role. For early breast cancer patients, the risk of recurrence and metastasis can be reduced; for advanced patients, it can alleviate the condition, prolong survival, and improve the quality of life.

After decades of development, the commonly used chemo-drugs for breast cancer currently include anthracyclines (such as DOX), taxanes (such as PTX), and antimetabolites (such as gemcitabine). DOX is an anti-tumor drug commonly used in clinic. Its main function is to insert the flat n-loop to the middle of the base strand of DNA, which prevents the transfer and replication of DNA and +RNA, thus avoiding cells' proliferation and metabolism. Breast cancer is a major indication of DOX, which is often used in combination with cyclophosphamide, in addition, it can be combined with PTX and docetaxel, which can increase the efficacy (Darya Alizadeh, 2014). In addition, DOX can be used to treat bladder cancer, head and neck malignancies, testicular malignancies, liver cancer, and stomach cancer.

PTX is a natural secondary metabolite extracted from the bark of Taxus chinensis, which has good antitumor effect. It is often used in the treatment of ovarian cancer, uterine cancer, and breast cancer. PTX is a natural secondary metabolite extracted from the bark of Taxus chinensis; it allows tubulin and tubulin dimers that make up microtubules out of dynamic equilibrium, which leads to them death of cells (Sun, 2008). DOX and PTX can be administered separately, but the therapeutic effect is not good. A single drug is easy to cause drug resistance. Once drug resistance occurs, the therapeutic effect of drugs will be significantly reduced, even large amounts of doses will not achieve the original effect. And improved drug dosage will lead to the relatively large toxicity and side effects. Therefore, we decided to use a Co-administration Combination therapy, refers to the use of two or more drugs for treatment, which can improve the efficacy and reduce adverse reactions. The combination of more drugs can reduce the dose of individual drugs, thus reducing the toxicity and side effects (Moussa, 2018). The combination of the two drugs can reduce the side effects and resistance significantly. PTX has the function of preventing the cell division form beginning of cell division cycle. DOX functions in cell DNA damage. It causes the cleavage of DNA and thus indirectly causes the generation of hydroperoxide through the oxidative reaction of NAD(P)H. Eventually, it leads to the apoptosis of cells (Hideki Mizutani, 2005). When combining the two drugs, the result suggested that it had become more effective on tumor regression. It can also reduce the side effects and the resistance (Gill, 2019). In order to improve the therapeutic effect of combined drug delivery and reduce its side effects, we decided to load these two drugs into liposome with. modifications of targeting and sustained release on liposomes.

Folate acid is applied for tumor targeting in our liposome system, although folate receptors are widely distributed in normal tissues and tumor tissues, the density and activity of folate receptors in most tumor cells are much higher than those in normal cells. Thus, it can achieve drug accumulation to the surface of breast cancer cells and increase the concentration of the drug in the tumor area, which can improve the treatment effect and reduce side effects. PH responsive drug release in tumor cell is achieved by DOBAQ, a kind of pH sensitive Cationic lipid, which exhibits pH dependent ionization and promote

liposome disruption in pH 5.5 endosome in tumor cell (Su, 2019). Because liposomes will dissociate and change with each other under different pH conditions, then control the drug release. Thus, we developed this way of delivering PTX and DOX. Using both drugs can limit the side effects. Additionally, in order to ease the cardiotoxin of those two drugs, we used pH-sensitive liposome to deliver the drug and only targeting on the breast cancer cells that has large amount of folate receptors (Sharma, 2006). The structure of liposome is shown in Figure 1. We hoped the bilayer liposome can delivering those two drugs effectively and reach our propose of reducing the side effects and lower the simple drug resistance. Therefore, we will find a more efficient way that can bring patient a better life quality and overall improve the survival rate.

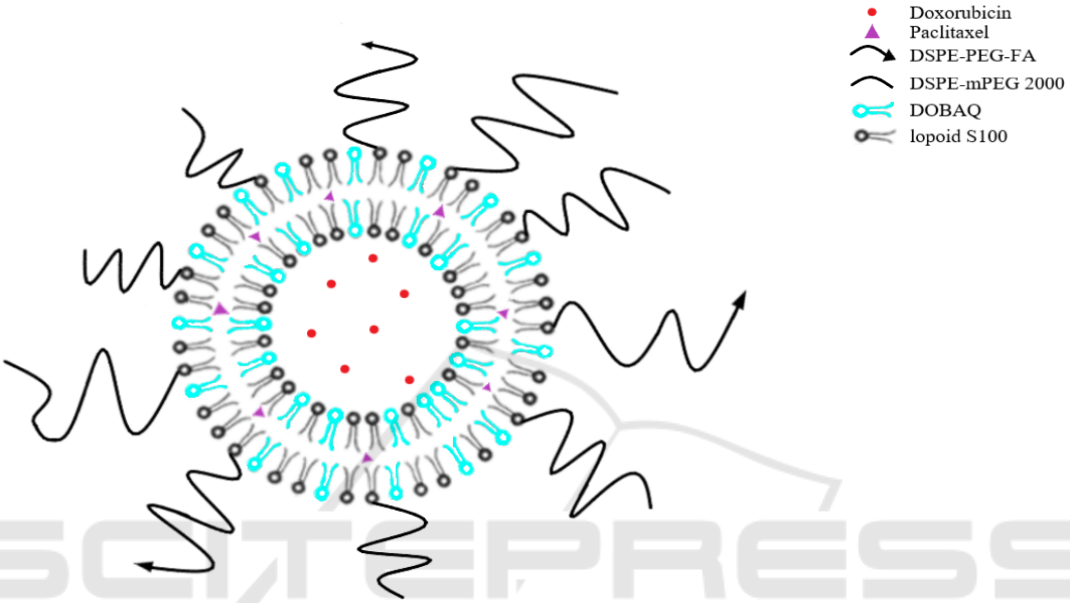

Figure 1. Schematic representation of the co-delivery of hydrophobic drug paclitaxel (purple) and hydrophilic drug doxorubicin (red) through bilayer liposome (Liu, 2014).

2 METHODS AND MATERIALS

2.1 Materials

DSPE-PEG (2000)-Folate and Doxorubicin hydrochloride (DOX) was purchased from Sigma-Aldrich (Shanghai) Trading Co.Ltd. (Shanghai, China). DOBAQ, DSPE-mPEG2000 and DSPE-PEG2000-FITC were purchased from Avanti Polar Lipids (Alabaster, USA). Lipoid S100 were purchased from Lipoid GmbH (Würzburg, German). Paclitaxel (PTX) were purchased from Macklin Inc. (Shanghai, China). Gibco™ PBS were purchased from Thermo Fisher Scientific (USA). Sephadex G-50 columns were purchased from Pfizer Inc. (USA). Amicon Ultra 0.5 mL centrifugal filters were purchased from Sigma-Aldrich (Shanghai) Trading Co.Ltd. (Shanghai, China). Pierce™ Protein Concentrator PES, 3K MWCO cut-off tubes, Attune NxT flow cytometry and Nunc™ multi-well cell culture plates were purchased from Thermo Fisher Scientific™. Leica SP2 CLSM Confocal laser scanning microscopy was from Leica Microsystems (Wetzlar, Germany).

2.2 Preparation of liposome

Liposomes were prepared by the lipid hydration method (Schiffelers, 2003). Briefly, different masses of lipid substances, were dissolved in round-bottomed vials using an organic solvent mixture (chloroform: methanol=2:1). Then, the round bottom vials are placed on the rotary evaporator. By using the principle of lowering the boiling point of liquids under reduced pressure, volatile solvents are removed by heating continuous distillation. After completion of the rotary evaporation operation, the resulting film in the vial was transferred to vacuum and dried for more than 6 hours to completely remove the residual organic solvent. The obtained films were placed in 2 mL of phosphate-buffered solution (PBS, pH 7.4) at 37°C for 30 min of hydration to obtain liposomes with

a final lipid concentration of 20 mg mL-1, followed by 60 seconds of sonication. Then it was further intermittently sonicated by a probe sonicator in ice-bath at 80 W for 75 s. The free (NH4)2SO4 was removed by passing through a Sephadex G-50 column in PBS (pH 7.4) solution. The obtained liposomes were purified by 3 K Amicon centrifugal filters and washed twice with fresh PBS solution (10 mM, pH 7.4).

For the preparation of liposomes containing DOX (D-LPs), we set up four concentration gradient groups (10 μg/ml, 1 μg/ml, 0.1 μg/ml, 0.01 μg/ml), and added gradient concentrations of DOX, stirred, and then incubated at 45℃ for 20 min. The PTX-loaded liposomes (P-LPs) were made at the same concentration and by the same method. By using gel filtration, the large molecule liposomes are eluted first due to their weak retention ability, while the small molecule compounds are retained strongly and exit the column last, thereby removing the free PTX/DOX. The liposomes were stored at 4℃ for later use.

For the preparation of DOX and PTX-loaded liposomes (DP-LPs), as shown in the Figure 1, the PBS was replaced by 1 ml 300 Mm (pH 4) (NH4)2SO4 solution. As mentioned above, the PBS solution (pH, 7.4) was added to the SephadexG-50 gel filtration column to equilibrate the pH, and then PTX-LPs were used to replace the outer phase consisting of (NH4)2SO4 solution. Doxorubicin was then remotely loaded using the (NH4)2SO4 gradient method. (Bolotin E M, 1994). Similarly, we set up four concentration gradient groups (10μg/ml, 1μg/ml, 0.1μg/ml, 0.01μg/ml) and the molar ratio of PTX to DOX was 1:1. Briefly, PTX-LPs were preheated at 50°C, and the appropriate amount of DOX solution was added and incubated with liposomes at 50°C for 20 min with gentle stirring to load DOX into the liposomal internal phase. Free DOX and PTX were then removed by 3 K Amicon centrifugal filters. Liposomes were stored at 4℃ and used within 24 h of preparation (Qiu, 2016). The Tables 1-3 list the lipid ratios of the liposomes used in the following experiments (Walsh, 2012; Moghimipour E, 2018; Campbell R B, 2001; Bernsdorff C, 1999; Sampedro F, 1994; Sharma A, 1994).

Table 1: Liposome formulation of FA-LPs.

	Corresponding compositions (%)			
	DSPE-PEG2000-Folate	DSPE-mPEG2000	DOBAQ	lipoid S100
Molar Ratio (%)	1	4	50	45

Table 2: Liposome formulation of FA-FITC-LPs for cell uptake study

	Corresponding compositions (%)				
	DSPE-PEG2000-Folate	DSPE-mPEG2000	DOBAQ	lipoid S100	DSPE-PEG2000-FITC
Molar Ratio (%)	1	4	49	45	1

Table 3: Liposome formulation of FITC-LPs for cell uptake study

	Corresponding compositions (%)			
	DSPE-PEG2000-FITC	DSPE-mPEG2000	DOBAQ	lipoid S100
Molar Ratio (%)	1	4	50	45

2.3 Physicochemical Characterization of Liposomes

For encapsulation efficiency measurement (Shew R L, 1985), 0.1 ml of the liposome suspension was passed through a Sephadex G-50 gel filtration column and the PTX and DOX contents were measured by high performance liquid chromatography (HPLC) at 227 nm and at Ex of 470 nm, Em of 590 nm, respectively. Drug entrapment efficiency (DEE, wt%) and drug loading capacity (DLC, wt%) were calculated according to the following formulas (Tang, 2014):

DEE = (amount of loaded drug/amount of drug added) × 100%

DLC = (amount of loaded drug/amount of loaded drug + amount of drug carrier) ×100%

The column used for high performance liquid chromatography (HPLC) is Acclaim™ 300 C18 column, flow rate is 1 mL/min, the liquid phase is of paclitaxel was methanol-water (65:35, v/v) with the detection wavelength of 227 nm and the liquid phase of adriamycin was methanol-water (70:30).

Hydrodynamic diameter, polydispersity index (PDI) and zeta potential of liposomes were measured by NS-90Z Nanoparticle size and potential analyzer from Omec (Zhuhai, China), using dynamic light scattering (DLS) and electrophoretic light scattering technique. The liposome was diluted with Milli Q water before measurement.

Transmission electron microscopy (TEM) samples were prepared by diluting the liposome solution to a concentration of 0.1 mg/mL and adding dropwise 5 μL of liposome dissolved into a 200-mesh formvar-coated copper grid (TABB Laboratories Equipment, UK). After five minutes, the solution was aspirated through filter paper and 2 μL of uranyl acetate solution (2%, w/v) was added. After five minutes, the solution was blotted off with filter paper. The TEM sample was air-dried and then assayed.

2.4 In Vitro Release Kinetic of Liposomes

Here, we used the dialysis method to investigate the release kinetics of DOX and PTX from liposomes in phosphate-buffered saline (PBS), a method that has been used several times in previous liposome delivery studies (Campbell R B, 2001; Lv, 2014; Wang, 2016) The pH of the PBS release medium was set to 7.4 and 5.5, respectively. Briefly, the liposomes loaded with PTX and Dox were suspended in 5 ml of PBS release medium and transfer to a dialysis bag (MWCO 3500 Da). The release experiments were started by placing the dialysis bag into 45 mL of release medium with continuous shaking at 100 rpm at 37°C. At the scheduled point in time (1,2,4,612,24,48,72,108 h), 4 mL of the incubation solution was extracted and replaced with an equal volume of fresh PBS. DOX release was determined using the HPLC method mentioned above. The concentration of paclitaxel in 1 mL of solution will be detected at 227 nm (flow rate 1 mL/min) on a C18 column. At the end of 108 h, the dialysis bag was opened and 2.0 ml of 10% TritonX-100 was mixed completely with the release medium. The concentrations of paclitaxel and Adriamycin were then determined by HPLC and UV-Vis spectrometer as mentioned above, and the maximum drug release was calculated.

After calculating the free drug concentration, the percentage of drug release can then be calculated with the following formula: percentage of drug release = amount of drug released / amount of drug contained in the package × 100%. The drug co-delivery release profiles of Dox and PTX have demonstrated that liposomes have slow linear sustained release kinetics and efficient drug loading yields. Drug release profiles at pH = 7.4 and 5.5 have also been clearly studied (Lv, 2014; Zhu, 2015). Based on previous studies, we predicted the release profiles of bilayer liposomes loaded with Dox and/PTX drugs administered in combination or alone at 1, 2, 4, 6, 12, 24, 48, 72, and 108 hours and compared them.

2.5 Stability Studies in Human Plasma

At 37°C, 10 mg of liposomes were added to 1 mL of human plasma and stirred at low speed (200 rpm) with a magnetic stirrer. After every period (1, 2, 4, 6, 12, 24h), the particle size of the liposomes was determined using dynamic light scattering. The liposomes were observed for the appearance of agglomerated precipitation.

2.6 Breast Cancer Cell and Liposome Interaction Studies

2.6.1 Cell Culture

Two types of human breast cancer lines, including MDA-MB-231 and MCF-7 were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose containing 10% fetal bovine serum (FBS), supplemented with 100 mg/ml streptomycin and 100 U/ml penicillin. The cells were maintained at 37°C in a humidified incubator under 5% CO2 (Qiu, 2016).

2.6.2 Confocal Laser Scanning Microscopy (CLSM) Observation of Liposome Uptake by Cells

The uptake of liposomes by both MDA-MB-231 and MCF-7 cells was determined by observing the fluorescence characteristics of DOX released from FITC-labeled liposomes (FITC-LPs) by confocal laser scanning microscopy (CLSM) at an emission wave of 480 nm and an excitation wave of 590 nm. Firstly, the cells were seeded on the coverslips in 24-well cell culture plates at a density of 1 ×105 cells/well in 2 mL of DMEM. Then the cells were incubated for 24 h to 50% confluence and the original medium was replaced with 200 ml of 3 mmol/ml DOX-PTX-FA-FITC-LPs (DP-FA-FITC-LPs) and DOX-PTX-FITC-LPs (DP-FITC-LPs). After 3 or 6 h incubation, then the supernatant was removed by centrifugation to obtain a cell mass. The cell masses were washed three times with cold PBS, fixed in 4% paraformaldehyde at room temperature for 30 min, followed by cell nuclei staining with DAPI for 5 min before washed three times with PBS for confocal microscopy analysis. Later, the coverslips were detected on CLSM. Fluorescence images of cells were analyzed using ImageJ or FIJI software (Lv, 2014).

2.6.3 Qualitative Analysis of Cellular Uptake Using Flow Cytometry

1.0 ml of MDA-MB-231 and MCF-7 cells (1×10^5 cells/well) were inoculated into 24-well tissue culture plates and cultured at 37°C, 5% CO_2 for 24 h until the cells grew almost confluently. The medium was then replaced with 1.0 ml of 0.5 mg/ml DP-FITC-LPs and DP-FA-FITC-LPs diluted with DMEM medium, respectively, and the plates were incubated at 37°C and 5% CO_2.After 4 h, the medium was removed, and cell monolayer was suspended by brief treatment with trypsin and then washed three times with cold PBS. Then the cell samples were examined by flow cytometry using a Attune NxT (Thermo Fisher). the level of cells that have taken up liposomes (positive event, %) and the level of liposomes that have been taken up (mean fluorescence) are measured. 10,000 sensor events collected and analyzed using FCS Express software (Qiu, 2016).

2.7 In Vitro Cytotoxic Studies

The cells were cultured as shown above. MDA-MB-231 and MCF-7 cells were plated at a density of 5×10^3 cells/well in 10% FBS-containing medium in 96-well plates and grown for 24 h. The cells were then exposed to liposomes (single drug/drug combinations; FA/without FA) at different concentrations of combined drugs or free drugs (10μg/ml, 1μg/ml, 0.1μg/ml, 0.01μg/ml), for 48 h. To determine the cytotoxicity of empty liposomes, 100 ml of 6 mmol/ml liposomes were added to each well of a 96-well plate and incubated with the cells described above. The cell viability was assessed using the CellTiter from Promega according to the manufacturer's instructions. The principle of detecting cell viability is that the fluorometric signal of CellTiter is proportional to the ATP content of the cells, which is proportional to the number of cells. Toxicity of each drug concentration was subsequently determined for each well. The data was analyzed by nonlinear regression to get the IC50 value. The combination index (CI) values were calculated by the equation:

$$\mathrm{CI} = \frac{C_{A,X}}{IC_{X,A}} + \frac{C_{B,X}}{IC_{X,B}} \quad (1)$$

CA,x and CB,x are the concentrations of drug A and drug B used in combination to achieve x% drug effect. ICx,A and ICx,B are the concentrations for single agents to achieve the same effect. A CI of less than, equal to, and more than 1 indicates synergy, additivity, and antagonism, respectively (Zhao, 2004). Using this analysis method, a CI = 0.9–1.1 reflects additive activity, and a CI >1.1 indicates antagonism, while a CI < 0.9 suggests synergy (Liu, 2014).

3 EXPECTED RESULTS AND CONCLUSIONS

3.1 Characteristics of Co-Delivery via pH Release FA Targeted Liposomes

Firstly, we characterized the physical properties of the dual- versus single-drug-loaded liposomes to determine whether the drug combination could alter the physical properties of the liposomal formulation. Dynamic light scattering (DLS) measurements showed that the resulting dual-loaded liposomes had similar mean hydrodynamic diameters as the single-loaded liposomes. Therefore, the effect of drugs on liposome particle formation is negligible (Qiu, 2016).

Next, we determined the encapsulation efficiency or loading yield of the liposomes. Particle size and PDI are important characteristics of drug-laden liposomes that determine the release kinetics of the drug, as shown in the table 4. Single- and dual-loaded

liposomes were dissolved in the release medium to gradually release the encapsulated drug (Dox and/or PTX). DOX and PTX concentrations were quantified by fluorophotometer and/or HPLC, respectively. The results of in vitro drug release assays showed that this liposome has slow and linear slow-release kinetics for DOX and PTX, similar to that of single drug liposomes. These results confirm the ability of the method to load different hydrophobic drugs into the same nanoparticles with efficient drug loading rates and sustained drug release profiles (Wang, 2016; Liu, 2014; Kurbacher C M,1996)

Table 4. Physico-chemical characterization of different liposomes formulations (Wang, 2016).

Sample	Particle size (nm)	PDI	Zeta potential (mV)	DLC of PTX (%)	DLC of DOX (%)	DEE of PTX (%)	DEE of DOX (%)
LP	126.8±3.3	0.14±0.02	+45.3±2.8	NA	NA	NA	NA
PTX LP	125.6±3.5	0.15±0.03	+37.6±2.6	18.6±2.0	NA	82.5±4.3	NA
DOX LP	128.3±3.4	0.12±0.03	+34.0±2.4	NA	10.3±1.6	NA	85.5±4.0
PTX-DOX LP	129.5±4.4	0.19±0.06	+26.8±3.3	12.5±1.8	9.6±1.2	81.7±4.6	83.6±4.3

* NA means not available.

3.2 In Vitro Release Kinetic of Liposomes

The DOX and PTX release behavior of liposomes was evaluated by phosphate-buffered saline (PBS) dialysis at different pH values (7.4 and 5.5). Among them, the release of DOX was strongly influenced by the ambient acidity. At pH 5.5, about 79% of DOX was released; at pH 7.0, less than 23% was released, as illustrated in Figures 2 and 3. To summarize, we present a robust combination chemotherapy approach that encapsulates two different types of antitumor therapeutics into a specific liposome formulation through a controlled 1:1 molar ratio. We demonstrated that liposomes could release Dox and PTX *in vivo* at predefined ratios of loaded drugs and induce synergistic effects in tumor cells. Such targeted reactive release liposomes have the superior ability to act as drug carriers, providing a controlled and sustainable spectrum of Adriamycin drug release with improved antitumor activity.

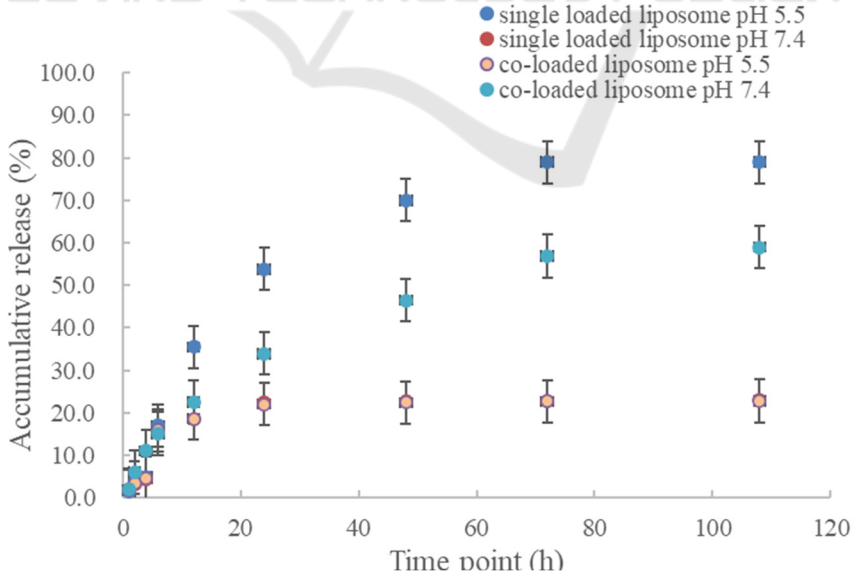

Figure 2: *In vitro* release of DOX in DOX loaded liposome and DOX/PTX co-loaded liposome at pH 7.4 and 5.5 buffer (Lv, 2014; Wang, 2014).

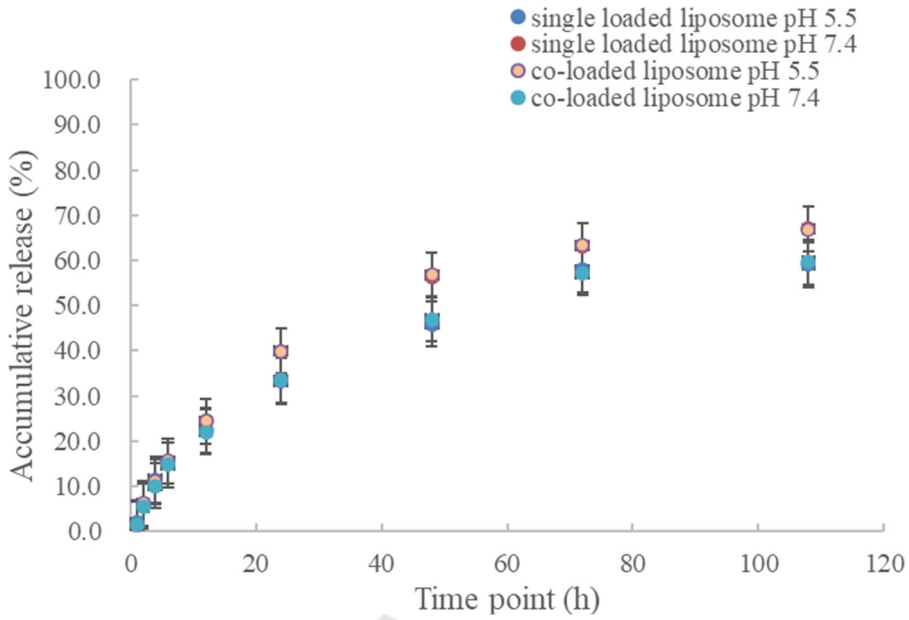

Figure 3: *In vitro* release of PTX in PTX loaded liposome and DOX/PTX co-loaded liposome at pH 7.4 and 5.5 buffer (Lv, 2014; Wang, 2014).

3.3 Stability Studies in Human Plasma

The particle size of liposomes was determined by dynamic light scattering at 37℃ for 24h to characterize liposome stability. No significant precipitation of liposomes with plasma proteins was observed, demonstrating good stability of liposomes.

3.4 Cellular Uptake Behavior of the Dual Drug Loaded Liposomes

The cellular uptake behavior of DP-FA-FITC-LPs in MDA-MB-231 and MCF-7 cells was studied using confocal laser scanning microscopy (CLSM). Nuclei were stained with DAPI (blue) and FITC (green) labeled liposomes were used for subcellular observation. After 3 h incubation, green fluorescence was observed in the cells. When the incubation period was increased to 6 h, the uptake of liposomes by the cells was enhanced and the green fluorescence was widely distributed in the cytoplasm, indicating that it could be successfully internalized by the tumor cells through endocytosis (Marinello P C, 2019)

3.5 In Vitro Cytotoxic Studies

The semi-inhibitory concentration values (IC50 values) for D-LPs and P-LPs were higher than those for free DOX and free PTX, respectively, as demonstrated in Tables 5 and 6. The reasons may be related to the different cellular uptake pathways of free drug and drug-loaded nanoparticles, as well as the different modes of controlled release of drug-loaded nanoparticles. In cell culture medium, most of the free drugs can show their effects rapidly after being transported into the cells by passive diffusion. In contrast, drug-loaded nanoparticles are mainly taken up by cells through the endocytic pathway and exert antitumor activity after the drug molecules are released from the nanoparticles.CI values below, equal to or above 1 indicate synergistic, additive or antagonistic effects, respectively. The calculated CI50 of free PTX or DOX were greater than one, indicating that they had no synergistic effect. Nanoparticles had a significant synergistic effect with CI50 values less than one, indicating that DOX and PTX coadministration was significantly better than free drug coadministration.

Table 5: IC50 of free drug and drug loaded liposomes on MCF-7 cell (Lv, 2014).

Samples	IC50 of PTX	IC50 of DOX	IC50 of combination
Free PTX	0.151±0.060	NA	NA
Free DOX	NA	0.132±0.055	NA
PTX LP	0.054±0.010	NA	NA
DOX LP	NA	0.049±0.012	NA
PTX-DOX LP	NA	NA	0.017±0.010

* NA means not available

Table 6: IC50 of free drug and drug loaded liposomes on MDA-MB-231 cell (Lv, 2014).

Samples	IC50 of PTX	IC50 of DOX	IC50 of combination
Free PTX	0.193±0.067	NA	NA
Free DOX	NA	0.176±0.069	NA
PTX LP	0.062±0.012	NA	NA
DOX LP	NA	0.059±0.009	NA
PTX-DOX LP	NA	NA	0.024±0.006

* NA means not available

4 CONCLUSIONS

In brief, we developed a liposome with folate-targeting and pH-responsive release capabilities for DOX and PTX co-delivery. It has sufficient structural stability, efficient delivery capacity, and good biocompatibility to show its potential to deliver antitumor drugs by intravenous injection. FITC-labeled this liposome can be absorb by MDA-MB-231 and MCF-7 tumor cells and has a synergistic inhibitory effect on tumor cells. It has high tumor accumulation, significant tumor suppression efficiency, and reduced systemic toxicity in vivo. Thus, our co-delivered liposomes are likely to achieve excellent results in the treatment of human breast cancer and also to provide additional design and value or combination therapy in other diseases. In the future, when the final dose-dependent response of the two anticancer drugs, the optimal concentration of anticancer effects, and the application of this strategy in the treatment of different tumors s are clearly investigated, clinics will be able to use lower concentrations of conventional drugs to provide better therapeutics and reduce patient suffering.

REFERENCES

Bernsdorff C, Reszka R, Winter R. Interaction of the anticancer agent Taxol™(paclitaxel) with phospholipid bilayers [J]. Journal of Biomedical Materials Research: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials, 1999, 46(2): 141-149.

Bolotin E M, Cohen R, Bar L K, et al. Ammonium sulfate gradients for efficient and stable remote loading of amphipathic weak bases into liposomes and ligandoliposomes [J]. Journal of liposome research, 1994, 4(1): 455-479.

Campbell R B, Balasubramanian S V, Straubinger R M. Influence of cationic lipids on the stability and membrane properties of paclitaxel-containing liposomes [J]. Journal of pharmaceutical sciences, 2001, 90(8): 1091-1105.

cOun, R., Moussa, Y. E., & Wheate, N. J. (2018). The Side Effects of Platinum-Based Chemotherapy Drugs: A Review for Chemists. *Dalton Transactions*, 47, 6645-6653.

Darya Alizadeh, Malika Trad, Neale T. Hanke, Claire B. Larmonier, Nona Janikashvili, BernardBonnotte, Emmanuel Katsanis and Nicolas Larmonier (2014). Doxorubicin Eliminates Myeloid-Derived Suppressor Cells and Enhances the Efficacy of Adoptive T-Cell Transfer in Breast Cancer. *Cancer Res*, 74 (1) 104-118.

Feng, Y., Ci, H., & Wu, Q. (2021). Expression of mammalian sterile 20-like kinase 1 and 2 and Yes-associated protein 1 proteins in triple-negative breast cancer and the clinicopathological significance. *Medicine*, 100(34), e27032.

Gill, J. H., Rockley, K. L., Santis, C. D., & Mohamed, A. K. (2019). Vascular Disrupting Agents in Cancer Treatment: Cardiovascular Toxicity and Implications for Co-Administration with Other Cancer Chemotherapeutics. *Pharmacology & therapeutics*, 202: 0163-7258.

G. Sharma, S. Anabousi, C. Ehrhardt & M. N. V. Ravi Kumar (2006). Liposomes as targeted drug delivery systems in the treatment of breast cancer. *Journal of Drug Targeting*, 14:5, 301-310.

Hassan, M. S. U., Ansari, J., Spooner, D., & Hussain, S. A. (2010). Chemotherapy for Breast Cancer. *Oncology Reports*, 24: 1121-1131.

Hideki Mizutani, Saeko Tada-Oikawa, Yusuke Hiraku, Michio Kojima, Shosuke Kawanishi (2005). Mechanism of apoptosis induced by doxorubicin through the generation of hydrogen peroxide. *Life Sciences*,76: 1439-1453.

Kurbacher C M, Wagner U, Kolster B, et al. Ascorbic acid (vitamin C) improves the antineoplastic activity of doxorubicin, cisplatin, and paclitaxel in human breast carcinoma cells in vitro[J]. Cancer letters, 1996, 103(2): 183-189.

Liu Y, Fang J, Kim Y J, et al. Codelivery of doxorubicin and paclitaxel by cross-linked multilamellar liposome enables synergistic antitumor activity[J]. Molecular pharmaceutics, 2014, 11(5): 1651-1661.

Liu Y, Fang J, Joo K I, et al. Codelivery of chemotherapeutics via crosslinked multilamellar liposomal vesicles to overcome multidrug resistance in tumor [J]. PLoS One, 2014, 9(10): e110611.

Lv S, Tang Z, Li M, et al. Co-delivery of doxorubicin and paclitaxel by PEG-polypeptide nanovehicle for the treatment of non-small cell lung cancer[J]. Biomaterials, 2014, 35(23): 6118-6129.

Marinello P C, Panis C, Silva T N X, et al. Metformin prevention of doxorubicin resistance in MCF-7 and MDA-MB-231 involves oxidative stress generation and modulation of cell adaptation genes[J]. Scientific reports, 2019, 9(1): 1-11.

Moghimipour E, Rezaei M, Ramezani Z, et al. Folic acid-modified liposomal drug delivery strategy for tumor targeting of 5-fluorouracil[J]. European journal of pharmaceutical sciences, 2018, 114: 166-174.

McGale, P., Correa, C., Cutter, D., Duane, F., Ewertz, M., Gray, R., Mannu, G., Pete, R., Whelan, T., Darby, S., & EBCTCG (Early Breast Cancer Trialists' Collaborative Group) (2014). Effect of Radiotherapy after Mastectomy and Axillary Surgery on 10-Year Recurrence and 20-Year Breast Cancer Mortality: Meta-Analysis of Individual Patient Data for 8135 Women in 22 Randomised Trials. *The Lancet*, 383(9935), 2127-35

Reddy, K. b. (2011). Triple-Negative Breast Cancers: An Updated Review on Treatment Options. *Current Oncology*, 18(4), 173-179.

Sampedro F, Partika J, Santalo P, et al. Liposomes as carriers of different new lipophilic antitumour drugs: a preliminary report [J]. Journal of microencapsulation, 1994, 11(3): 309-318.

Sharma A, Straubinger R M. Novel taxol formulations: preparation and characterization of taxol-containing liposomes [J]. Pharmaceutical research, 1994, 11(6): 889-896.

Shew R L, Deamer D W. A novel method for encapsulation of macromolecules in liposomes [J]. Biochimica et Biophysica Acta (BBA)-Biomembranes, 1985, 816(1): 1-

Sun, J., Duan, J., Dai, S., Ren, J., Guo, L., Jiang, W., & Li, Y. (2008). Preparation and Anti-Tumor Efficiency Evaluation of Doxorubicin-Loaded Bacterial Magnetosomes: Magnetic Nanoparticles as Drug Carriers Isolated from Magnetospirillum Gryphiswaldense. *Biotechnology and Bioengineering,* 101: 1313-1320.

Su, Z., Resend-ochir, T., Ganbold, T., & Baigude, H. (2019). Design of Curdlan-Based PH-Sensitive Polymers with Endosome Buffering Functionality for SiRNA Delivery. *Biological Macromolecules*, 146: 0141-8130.

Schiffelers R M, Koning G A, ten Hagen T L M, et al. Anti-tumor efficacy of tumor vasculature-targeted liposomal doxorubicin[J]. Journal of Controlled Release, 2003, 91(1-2): 115-122.

Trayes, K. P., & Cokenakes, S. E. h. (2021). Breast Cancer Treatment. *Am Fam Physician*, 104(2):171-178.

Walsh C L. Design, Synthesis, and Characterization of Novel Zwitterionic Lipids for Drug and siRNA Delivery Applications[M]. University of California, San Francisco, 2012.

Wang Y, Zhang H, Hao J, et al. Lung cancer combination therapy: co-delivery of paclitaxel and doxorubicin by nanostructured lipid carriers for synergistic effect[J]. Drug delivery, 2016, 23(4): 1398-1403.

W. j. g., Bodei, L., Giammarile, F., Maecke, H. r., Tennvall, J., Luster, M., & Brans, B. (2007). Targeted Therapy in Nuclear Medicine—Current Status and Future Prospects. *Annals of Oncology*, 18: 1782-1729.

Yin, W., Di, G., Liu, G., Wu, J., Lu, J., Han, Q., Shen, Z., Shao, Z. (2010). Clinicopathological features of breast cancer patients with nipple discharge. Molecular Medicine Reports 3.5: 863-868.

Yuan M, Qiu Y, Zhang L, et al. Targeted delivery of transferrin and TAT co-modified liposomes encapsulating both paclitaxel and doxorubicin for melanoma [J]. Drug delivery, 2016, 23(4): 1171-1183.

Zhu Y, Wang M, Zhang J, et al. Improved oral bioavailability of capsaicin via liposomal nanoformulation: preparation, in vitro drug release and pharmacokinetics in rats[J]. Archives of pharmacal Research, 2015, 38(4): 512-521.

Zhao L, Wientjes M G, Au J L S. Evaluation of combination chemotherapy: integration of nonlinear regression, curve shift, isobologram, and combination index analyses [J]. Clinical cancer research, 2004, 10(23): 7994-8004.