

Proceedings

# Folic Acid—Targeted Doxorubicin drug Delivery System for Triple-Negative Breast Cancer Treatment

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**Abstract:** Triple-negative breast cancer (TNBC) is a highly aggressive type of cancer with limited therapeutic options. However, this type of cancer cells have shown overexpression of folate receptors, which bind with folic acid (FA) with high affinity. This feature can be used for targeting of nanocarriers, such as liposomes. In order to further examine the potential of increased efficacy by targeting the folate receptor, we prepared folate conjugated liposomes (DSPC/Chol/PEG/DSPE-PEG-FA) and loaded them with doxorubicin (DOX), an anticancer drug. For this, we first synthesized and verified the conjugate between FA and PEG-lipid (FA-PEG-lipid). After that, liposomes were prepared with thin film hydration method followed by probe sonication. Three different types of targeted liposomes having different concentrations of FA-PEG-Lipid in their membranes (0.1 mol%, 0.5 mol% and 1mol%) were evaluated for their cytotoxicity (DOX-loaded liposomes) on MDA-MB-231 (epithelial human breast cancer cells), 4T1 (murine mammary carcinoma cells) and MCF7 (Human breast cancer cells); the two first are TNBC cells and overexpress FA receptor, the third does not. Cytotoxicity results proved that increasing amounts of FA on the surface of liposomes results in enhance antitumor activity of liposomal-DOX in the case of the cancer cells, which overexpress the FA receptor.

**Keywords:** liposomes; folic acid; doxorubicin; breast cancer; TNBC

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## 1. Introduction

Breast cancer (BC) is the most common malignancy and leading cause of cancer-related mortality among women worldwide [1,2]. Triple-negative breast cancer (TNBC) owes 15–20% of all the invasive subtypes of breast cancer [3] and is characterized by absence of expression of estrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor receptor 2 (HER-2) on tumors cell membrane. This feature eliminates the benefits of endocrine therapy and treatment, therefore mainly relies on chemotherapy [4]. Even systemic chemotherapy with clinically approved drugs reflects poor response, high toxicity and develops multidrug resistance. However, this type of cancer cells has shown overexpression of folate receptors, which bind with folic acid (FA) with high affinity. Folate receptor (FR)-a is a glycosyl phosphatidylinositol-anchored membrane protein that is selectively overexpressed in >90% of ovarian carcinomas [5], and to various extents in other epithelial cancers, but is only minimally distributed in normal tissues [5,6]. Therefore, liposomes conjugated to the folate ligand via a polyethylene glycol (PEG) spacer have been used to deliver chemotherapeutic agents, oligonucleotides, and markers to FR-bearing tumor cells.

Doxorubicin (DOX), an anthracycline antibiotic, is one of the most commonly used anticancer agents for the treatment of various types of cancers, including breast cancer. It intercalates between base pairs of the DNA helix, thereby preventing DNA replication and ultimately inhibiting protein synthesis. Herein, we used DOX as an anticancer drug, to examine the potential of increased efficacy of DOX-liposomes by targeting the folate receptor. For this, we first synthesized and verified the conjugate between FA and PEG-lipid (FA-PEG-lipid). After that, we prepared liposomes with three different concentrations of FA-PEG-lipid in their membranes, loaded them with DOX and compared their toxicity.

## 2. Experiments

### Materials

Folic Acid and Dicyclohexylcarbodiimide (DCC) were purchased from Sigma-Aldrich. 1,2-Distearoyl-sn-glycerol-3-phosphatidyl-choline [DSPC], and 1,2-Distearoyl-sn-glycerol-3-phosphatidyl-ethanolamine-*N*-[methoxy(polyethylene-glycol)-2000] [PEG2000] were purchased from Lipoid, Germany. Cholesterol (Chol) was purchased from Sigma-Aldrich (Darmstadt, Germany). DSPE-PEG-NH<sub>2</sub> was purchased from Avanti Polar Lipids. Doxorubicin, hydrochloric salt (DOX) was purchased by Tocris Bioscience, UK. Fetal Calf Serum (FCS) was from Sigma (Darmstadt, Germany).

A bath sonicator (Branson) and a microtip-probe sonicator (Sonics and Materials, Leicestershire, UK) were used for liposome preparation. Protein concentrations were measured by Bradford microassay. The Cell Viability MTT assay was carried out with 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (purchased by Sigma-Aldrich). All other reagents and chemicals used were of analytical grade and were purchased from Sigma-Aldrich.

### Methods

#### 2.1. Synthesis of DSPE-PEG2000-FA Lipid

Briefly, 12.65 mg of folic acid was dissolved in 1.2 mL and 0.208 mL of pyridine was added. Next, 40 mg of DSPE-PEG2000-amine were added to the mixture and 13 mg DCC dissolved in 0.19 mL DMSO. The reaction mixture was left to stir in a magnetic stirrer at room temperature overnight (according to TLC). 11 milliliters of water were added to the reaction mixture and the insoluble materials were removed by centrifugation at 15,000 g for 15 min. The supernatant was dialyzed 2 times in 50 mM NaCl for 24 h and then against water for 24 h. The dialyzed product (folate-PEG-DSPE) was lyophilized and stored at -20 °C. The formation of folate-PEG-DSPE was confirmed by reverse-phase high-pressure liquid chromatography and by H-NMR.

#### 2.2. Liposome Preparation and DOX Loading

Liposomes of the following compositions were used in this study: (a) folate-PEG-liposomes composed of DSPC/cholesterol/DSPE-PEG2000-MeO/folate-PEG-DSPE (61:30,5:8:0,1), (b) folate-PEG-liposomes composed of DSPC/cholesterol/DSPE-PEG2000-MeO/folate-PEG-DSPE (61:30,5:8:0,5), (c) folate-PEG-liposomes composed of DSPC/cholesterol/DSPE-PEG2000-MeO/folate-PEG-DSPE (61:30,5:8:1) and (d) control liposome not targeted with folic acid, composed of DSPC/cholesterol/DSPE-PEG2000-MeO (61:30,5:8). All liposome types were prepared by the thin-film-hydration method followed by probe sonication for size reduction. The resulting lipid films were hydrated at 60 °C with ammonium sulfate solution (120 mM, pH 5.5, 300 mOsm), for DOX loading; For physicochemical characterization, liposomes were hydrated with PBS buffer (pH 7.40, 300 mOsm). Vesicle size was reduced by subjection to a high intensity (750 Watt) probe sonicator (Sonics and Materials, Leicestershire, UK) for 15 min and then left at 60 °C for 1 h, to anneal any structural defects. DOX was loaded in LIP and FA-LIP by the active loading method, as described in detail elsewhere [7]. In brief, the vesicles formulated in ammonium sulfate ([NH<sub>4</sub>]<sub>2</sub>SO<sub>4</sub> (120 mM)) were

resuspended in PBS pH 7.4, for exchange of the dispersion media, and incubated (at a lipid concentration of 2.4 mg/mL) with 0.0887 mg/mL DOX solution (in PBS) (corresponding to a lipid/DOX ratio equal to 27:1 [*w/w*]), for 60 min at 60 °C. Lipid content of liposomes was measured by the Stewart assay, a colorimetric method used routinely for the quantification of phospholipids [8]. Liposomes were purified from non-encapsulated DOX by repeated ultracentrifugations for 1 h (each) at 60,000 rpm (Sorvall WX90 Ultra, Thermo Scientific, Waltham, MA, USA).

### 2.3. Physicochemical Properties of Liposomes

Liposome were characterized for their lipid concentration, mean diameter, size distribution, and zeta-potential. Vesicle size distribution was measured by dynamic light scattering (Malvern Instruments, Zetasizer Nano SZ, Malvern, UK). Sizes reported correspond to the z-average means of the hydrodynamic diameters of the liposomes. For  $\zeta$ -potential values, the electrophoretic mobility of the liposome dispersions was measured at 25 °C, by the same instrument. Zeta potential values were obtained (by the instrument) from the electrophoretic mobility, according to the Smoluchowski equation.

The final D/L (drug/lipid) ratio (*w/w*) was compared with the initial one, for calculation of the DOX encapsulation efficiency (%) of each liposome type, according to Equation (1):

$$EE (\%) = \frac{D/L_{final}}{D/L_{initial}} \cdot 100 \quad (1)$$

### 2.4. Cell Culture Studies

Four cell lines were used in this study: (i) MDA-MB-231 (epithelial human breast cancer cells), (ii) 4T1 (murine mammary carcinoma cells), (iii) MCF7 (Human breast cancer cells) and (iv) HEK (Human HEK-293 embryonic kidney cells). Cancer cells were grown in RPMI 1640 medium supplemented with 10% FBS and 1% antibiotic-antimycotic and the normal cells were grown in DMEM medium supplemented with 10% FBS and 1% antibiotic-antimycotic solution (Invitrogen, Carlsbad, CA, USA). The cells were cultured at 37 °C, 5% CO<sub>2</sub>/saturated humidity. Medium was changed every 2–3 days.

The toxicity of the various types of liposomes towards cancer and normal cells was evaluated by the corresponding reduction of cell viability after 24 h incubation with test (DOX-FA-LIP) or control formulation (DOX-LIP), by the MTT assay. For each experiment, cells were seeded overnight at 37 °C at a density of  $5 \times 10^4$  cells per well, in 24-well plates and then incubated for 24 h at 37 °C (5% CO<sub>2</sub>/saturated humidity) with 0.4 mL growth medium and 0.1 mL of the formulation evaluated in each case. Cell viability (%) was calculated by the equation (2):

$$Viability (\%) = \frac{OD-570_{sample} - OD-570_{background}}{OD-570_{control} - OD-570_{background}} * 100 \quad (2)$$

where, OD-570<sub>control</sub> corresponds to untreated cells (or PBS control) and OD-570<sub>background</sub> to MTT without cells. All samples were diluted in order to achieve DOX concentrations of 3  $\mu$ M.

## 3. Results

### 3.1. Physicochemical Properties and Stability of FA-LIP and LIP

All liposome dispersions were characterized for their size distribution (mean hydrodynamic diameter and polydispersity index), and their zeta-potential (Table 1). It should be pointed out that the liposomes characterized were formulated in PBS buffer, pH 7.40.

**Table 1.** Physicochemical properties of FA-LIP, and LIP, formulated in PBS buffer (pH 7.40).

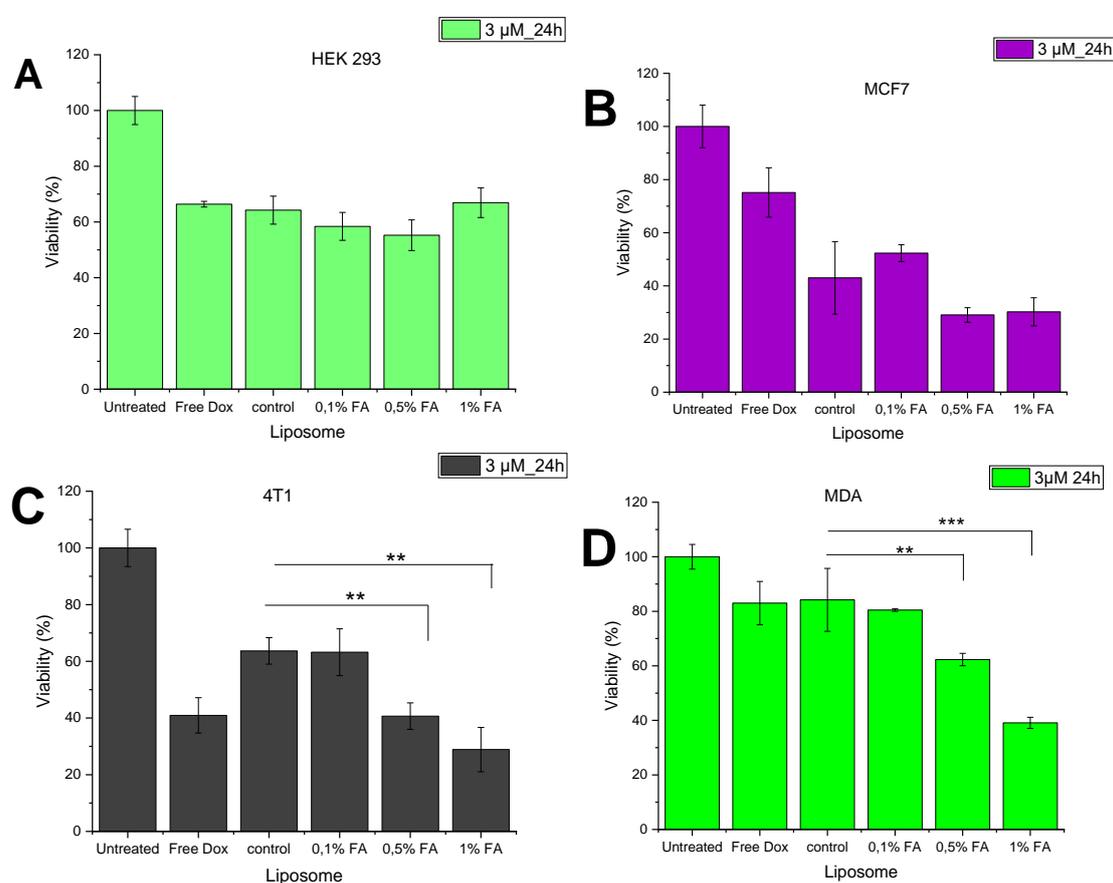
DSPE-PEG2000-FA (Mole%)	Mean Hydrodynamic Diameter (nm)	PDI	$\zeta$ -Potential (mV)	DOX Loading (%)
0	99.7 $\pm$ 3.05	0.243	-5.49 $\pm$ 0.33	91.4 $\pm$ 2.2
0.1	99.9 $\pm$ 1.4	0.159	-4.38 $\pm$ 0.91	94.1 $\pm$ 1.7
0.5	124.1 $\pm$ 0.7	0.192	-7.4 $\pm$ 1.6	95.9 $\pm$ 2.2
1	126.5 $\pm$ 2.4	0.161	-5.7 $\pm$ 1.1	97.3 $\pm$ 2.3

As seen in Table 1, the mean hydrodynamic diameter of all the liposomes prepared was in the nano-range, between 99 and 127 nm. The polydispersity index values were between 0.159 and 0.243. The addition of FA-lipid was shown to increase slightly the mean diameter of the vesicle and more precisely, a higher molar ratio of the lipid incorporated is connected with higher diameter respectively. Zeta potential values were in the range between -4.38 and -7.41 mV.

Additionally, the loading of DOX achieved in all liposome types ranged between 91% and 97%. The addition of FA had no significant effect on DOX loading, in comparison with the untargeted liposome.

### 3.2. In Vitro Anticancer Activity

As seen in Figure 1, incubation of HEK (Figure 1A) and MCF-7 cells (Figure 1B), that do not overexpress the FA receptor, with targeted DOX liposomes (at the same DOX concentration), does not seem to have any effect to enhance the anticancer activity of the non-targeted DOX-liposomes (control), as anticipated. Opositely, the targeted liposomes seem to enhance the anticancer activity of DOX, compared to the control liposomes, in the cases of cells which overexpress the FA receptor, such as the 4T1 cells (Figure 1C), and the MDA-MB-231 cells (Figure 1D). The later effect is seen only when the FA density on the targeted liposomes is higher that 0.5% and especially in the case of the liposomes that have 1 mole% of FA-conjugated PEG-lipid in their membranes.



**Figure 1.** Viability of cells after incubation with FA-targeted DOX-LIP and control (non-targeted) liposomes and free DOX, corresponding to a DOX concentration of 3  $\mu\text{M}$  for 24 h (A) HEK cells; (B) MCF-7 cells. (C) 4T1 cells and (D) MDA-MB-231 cells. \*\* and \*\*\* correspond to  $p < 0.01$  and  $p < 0.001$  and respectively.

#### 4. Discussion

Herein, we investigated the potential to target DSPE-Chol-PEG liposomes with FA and its effect on the anticancer properties of DOX. Furthermore, we evaluated the ability to load FA-LIP with DOX using the active loading method, in order to develop potential improved anticancer chemotherapeutic systems. All formulations of FA-LIP as well as LIP (which were prepared as control vesicles), had mean diameters in the nano-range, between 99 and 127 nm (Table 1), polydispersity values below 0.250, and slightly negative zeta potential values. Concerning the capability of using the active loading protocol to load DOX into FA-LIP, the current results confirmed that high amounts of DOX can be loaded into FA-LIP successfully.

The in vitro experiments carried out with DOX-FA-LIP on HEK, MDA-MB-231, MCF7 and 4T1 cells demonstrated interesting anticancer potential of DOX-FA-LIP with 1mole % r of FA-PEG-lipid in their membranes, toward the cancel cell types that over express FA receptor (Figure 1).

Preliminary FITC-uptake studies indicate that the targeting potential of the current liposomes is low. Perhaps this may be linked with the fact that FA was attached on lipid-PEG conjugates with PEG 2000 molecules; the use of higher MW PEG to better expose FA on the liposome surface has been proposed by others, as a method for enhancing targeting potential. Currently we are evaluating the effect of the PEG MW on the targeting potential of FA-targeted liposomes.

#### 5. Conclusions

In this study, folic acid-targeted DSPE-PEG lipid was successfully synthesized and utilized as a homing ligand to target DOX to cancer cells, which overexpress the FA receptor. Our data show that liposomes with DSPE-PEG-FA can efficiently load DOX and form stable nano assemblies with a size within the nanoscale. Folic Acid as a ligand on liposome surface can improve the antitumor activity of liposomal DOX towards Triple-negative breast cancer (TNBC) cells; we are currently evaluating methods to further optimize the targeted DOX-liposomes.

**Author Contributions:** S.G.A. conceived the experiments and got the funding. S.M. designed the synthesis of DSPE-PEG-FA lipid and the molar composition of each liposome. A.M. conceived the experiments regarding the cells. F.G. designed and helped with the execution of the cell experiments. E.L performed all the experiments needed together with M.M.; E.L. wrote a first draft of the paper. E.L., F.G. and M.M. gathered and analyzed the data. S.A. corrected the draft paper and wrote the final version.

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**Conflicts of Interest:** The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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