

Novel combinational nanomedicines, liposomes, to tackle breast cancer

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Abstract

Doxorubicin (DOX), a potent chemotherapeutic, is a commonly prescribed treatment for breast cancer, but is limited by severe organ toxicity. Therefore, more effective therapies are required. Aims: this study developed a novel DOX liposomes (LipDOX-ALA-AA) co-loaded with alpha-lipoic-acid (ALA) and ascorbic-acid (AA) to enhance antineoplastic effect. Methods: liposomes were fabricated using a microfluidic system with a DSPC lipid:Cholesterol ratio of 1:1 and a flow rate ratio of 5:1. Liposomes were investigated using various techniques such as dynamic light scattering to measure liposomes' size and charge; UV-spectroscopy to determine DOX-encapsulation-efficiency, EE, and drug release. Cytotoxicity assays used various cell-lines. Results: data revealed that LipDOX-ALA-AA had diameter of 79.0 ± 0.3 nm, with narrow size distribution (polydispersity was 0.1 ± 0.01), and zeta-potential of -4.0 ± 1.2 . DOX EE exceeded 95%, drug load was 0.5 mg/105.5 mg total content, drug release followed a biphasic pattern. Cytotoxicity assay showed significant activity ($p < 0.05$) against breast cancer cell-lines with reduced nephrotoxicity compared to commercial Doxosome. Conclusion: this novel formulation (LipDOX-ALA-AA) offers a promising advancement in breast cancer therapy.

Keywords: liposomes; reactive oxygen species; doxorubicin; antioxidants; alpha-lipoic acid; ascorbic acid; apoptosis; breast cancer

Introduction

Liposomes are spherical-shaped vesicles consisting of one or more phospholipid bilayers. Liposomes have been proven as a suitable candidate for drug delivery in nanomedicine due to their small vesicle size, biocompatibility, nontoxic, non-immunogenic, and biodegradable natures (Elkordy *et al.*, 2024). When phospholipids molecules are exposed to aqueous media, they tend to form a self-assembled bilayer structure by the attraction of polar head groups to the aqueous media and repellent nonpolar tails from aqueous media. (Akbarzadeh *et al.*, 2013; Daraee *et al.*, 2014). Liposomes have shown remarkable utility in recent years, becoming a convenient drug delivery system for cancer treatment within the nanomedicine field because of their advantageous features (Attia *et al.*, 2020, Attia *et al.*, 2021).

Small sized liposomes loaded with anticancer drugs can provide an efficient means for targeting tumour cells. The characteristics of tumour vasculatures have the potential to improve the transportation of liposomes through the enhanced permeability and retention (EPR) effect. This biological process enables nanoparticles smaller than the pore size in the tumour vasculature to escape and, move into

the tumour interstitium via the leaky tumour vasculature and molecular movement within fluids and finally accumulate in the perivascular regions thus improving the passive targeting of liposomes (Attia *et al.*, 2020). Consequently, the application of liposomes has led to a higher cellular uptake that reduced drug dose and increased the systemic circulation half-life. Several methods have been developed to produce small sized liposomes. Conventional methods for liposomes production include but not limited to; thin-film hydration, solvent injection, reverse-phase evaporation, electro-formation and detergent dialysis, among others (Akbarzadeh *et al.*, 2013; Pandey *et al.*, 2016; Nkanga *et al.*, 2019). However, the novel microfluidic system offers significant advantages over the traditional methods. Microfluidic devices can rapidly produce liposomes from a few millilitres to clinical scales of several litres in reproducible, and cost-effective manners. It can produce liposomes in a one step process with homogenous particle size. In addition, it provides a high level of control over the processing parameters leading to enhanced level reproducibility and robustness (Joshi *et al.*, 2016; Forbes *et al.*, 2019; Shah *et al.*, 2020).

Breast cancer is the second cause of mortality in females and one of the most common types of cancer (Saha *et al.*, 2021). Globally, breast cancer accounts for 1 in 8 cancer diagnoses (Arnold *et al.*, 2022). Further, breast cancer was ranked as the fifth leading cause of cancer death cases globally, contributing to 685,000 deaths. Amongst females, 1 in 4 women are diagnosed with breast cancer while 1 in 6 women dies of breast cancer (Sung *et al.*, 2021). Despite the enhanced effect of using chemotherapies in cancer treatment, chemotherapy still exert long-term sequelae and side effects which is considered as a main source of concern (Nurgali *et al.*, 2018). For instance, cisplatin and doxorubicin are two of the most potent chemotherapy medications used to treat various types of cancer. High doses of both drugs are more efficacious for the suppression of cancer; however, their administration was associated with numerous side-effects including but not limited to fatigue, alopecia, nausea and vomiting, oral sores, nephrotoxicity, myelosuppression, ototoxicity, and neurotoxicity. (Barabas *et al.*, 2008; Aldossary, 2019; Songbo *et al.*, 2019; Ajaykumar, 2020).

Doxorubicin (DOX) is considered as one of the most potent cytotoxic drugs for breast cancer treatment but exhibits significant side effects with high level of organ toxicity. For instance, DOX treatment requires limiting the cumulative lifetime dose to avoid the risk of developing cardiomyopathy (Christowitz *et al.*, 2019; Liu *et al.*, 2019). The antitumor effect of DOX significantly increases as dose increases. Nevertheless, its therapeutic value and clinical application are hindered due to several factors such as its hydrophilic nature [as HCl salt], short half-life, low bioavailability, and high volume of distribution. Additionally, antitumor effectiveness of DOX is mainly observed when administered at high doses which in turn results in significant adverse side effects like cardiotoxicity, extravasation, nephrotoxicity, myelosuppression, gonadotoxicity, and renal toxicity (Mohan *et al.*, 2021; van der Zanden *et al.*, 2021; Johnson-Arbor and Dubey, 2022; Peter *et al.*, 2022). Moreover, DOX induced cellular and organ toxicities that are caused by the elevation of reactive oxygen species (ROS), reactive nitrogen species and antioxidant depletion had led to oxidative stress that may activate cytotoxic signalling for destruction of DNA, proteins and lipids which eventually caused cellular damage and death (Cappetta *et al.*, 2017; Aldossary, 2019; Kullenberg *et al.*, 2021).

Another major challenge for preparing a stable anti-cancer drug is the chemical instability, short half-life and poor solubility of cytotoxic agents (Chidambaram *et al.*, 2011; Cheng *et al.*, 2021). The main degradation pathways are oxidation and hydrolysis. Hydrolysis occurs in aqueous media; therefore, the injectable chemotherapeutics are mostly in solid lyophilised forms to be reconstituted before administration, meaning that extra steps are necessary before injections (Olusanya *et al.*, 2018; Mao *et al.*, 2019). For examples, daunorubicin, doxorubicin, vinblastine, chlorambucil, vincristine, irinotecan and melphalan, have been found to be highly unstable in milli-Q water (pH of 6.3) due to the presence of reactive groups in their chemical structures, which favour hydrolytic reactions. In particular, daunorubicin, doxorubicin, irinotecan and vincristine have rapidly degraded, and only 10% of the initial concentration has been detected after 5 min of exposure to room temperature and neutral pH (Ioele *et al.*, 2022). The hypothesis of this work postulates that the use of novel microfluidic technique will enable the achievement of rapid, cost-effective, and highly reproducible liposome production, from small-scale in millilitres to clinical volumes in litres when compared to conventional methods. By employing microfluidic technology, the project aims to streamline the process into a one-step preparation of liposomes, ensuring homogenous particle sizes and high level of vesical stability without the use of post-production processing such as homogenisation, extrusion and lyophilisation.

In this project, the formulation of liposomes will incorporate a combination of three active compounds (alpha lipoic acid, ascorbic acid, and doxorubicin) that possess anticancer properties into a single liposomal vesicle to elicit a synergistic effect. The optimised liposomes are expected to synergise the antineoplastic effect, increase drug release and drug efficiency ex-vivo, hence, expected to decrease the pharmacological dose which consequently reduce the risk of toxicity and adverse effect, initiate a chemoprotective effect against the heart and kidney and finally reduce the cost, and overcome the significant side effects of conventional therapies.

1 Experimental section

1.1 Materials

The lipid, 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) was obtained from Lipoid, Germany. Cholesterol (Chol) $\geq 99\%$, Ascorbic Acid (AA), Alpha Lipoic Acid (ALA), crystal violet (CV) solution, Doxorubicin hydrochloride (DOX), Ethyl alcohol Absolute (Eth), Iso-propyl alcohol, Phosphate buffer saline tablets pH 7.4, 2',7'-Dichlorofluorescein diacetate were all purchased from Sigma-Aldrich (Gillingham, UK). Doxosome™-Doxorubicin Liposomes (PEGylated) was purchased from Encapsula NanoSciences (USA).

1.2 Liposome Preparations

1.2.1 Liposomes Experimental Design Plan

DSPC was used to prepare several liposomes with different parameters (lipid to cholesterol ratios (1:1 and 2:1), microfluidic flow rate ratios of aqueous to organic phases (1:1, 2:1 and 5:1)) and total flow rate (12 mL/min) for preliminary studies to find the most optimised formulation regarding the physicochemical properties (vesicle size, zeta potential (ZP) and encapsulation efficiency). Afterwards, the formulations that showed the best parameters were used to formulate liposomes coloaded with AA then, crystal violet (CV) was used as a model water soluble drug, before using DOX, to measure the

encapsulation efficiency and investigate whether the CV will influence the liposomal vesicle size along with AA. A final liposomal preparation containing DOX, AA, and ALA was prepared based on the obtained preliminary results as summarised in section 1.2.2 and Table 1.

The rationale for the choice of the lipid is that DSPC is a neutral phospholipid and has been used in this study for liposome formulations since it mimics the biological membrane in many aspects, being one of their most abundant constituents (Shafaa, 2020). In addition to its high phase transition temperature which provide high structural stability for liposomes and lipid nanoparticles (Cheng and Lee, 2016). Additionally, lipid-to-cholesterol ratios and microfluidic flow rates and DSPC lipid concentration were chosen based previous studies, producing liposomal formulations with small and narrow size distribution and high drug entrapment efficiency. DSPC aligns with standard methodologies in liposome research, ensuring reproducibility and reliability in the formation of stable liposomal formulations, where increasing DSPC concentration (0.3–10 mg/mL) showed a reduction in liposomes size (Forbes et al., 2019). While the lipid-to-cholesterol ratios were chosen based on preliminary studies conducted in this research, which indicated a smaller liposome mean diameter and enhanced stability.

All experiments were performed in triplicates (n=3).

Insert Table 1

1.2.2 Liposome Preparations Using Microfluidic System

Liposomes were formulated using the NanoAssemblr benchtop machine provided by Precision NanoSystems Inc. (Vancouver, Canada) according to the Precision Nano Systems protocol). The liposomal formulations were prepared with different lipid:cholesterol ratios (1:1 and 2:1) and flow rates ratios (FRR) (1:1, 2:1 and 5:1)) using the microfluidic system with a fixed total flow rate of 12 mL/min. The concentration of lipid: cholesterol ratio (1:1) and (2:1) was (2.5:2.5 mg/mL) and (5:2.5 mg/mL), respectively.

Drug concentrations used for DOX, AA, ALA, and CV were 0.5, 50, 50 and 0.05 mg/ mL, respectively. CV, DOX and/or AA were dissolved in the aqueous buffer (PBS pH 7.4) to form the aqueous phase. Cholesterol, DSPC and/or ALA were dissolved in the non-aqueous medium (ethanol) to form the organic phase. Then both aqueous and organic phases were loaded onto the microfluidic cartilage to produce blank and drug containing liposomes. The composition of liposomal formulation was presented in Table 1.

1.3 Liposomes Physicochemical Characterisation

1.3.1 Dynamic Light Scattering

The size distribution, mean diameter, and polydispersity index (PDI) of the liposomes were measured by dynamic light scattering (DLS) on a Zetasizer Nano-ZS (Malvern Instruments Ltd., UK). While zeta potential was determined by using laser Doppler electrophoresis technique, measurements using the M3-PALS method in disposable folded capillary cells using the same device. Measurements were made at 25°C with an angle of scatter of 173°. The liposomes were diluted 10 folds with PBS pH 7.4 prior to measurement. They were filtered twice using syringe filter 0.45 µm to avoid any potential contaminants that could influence the measurement accuracy.

1.3.2 Determination of Encapsulation Efficiency

Entrapment efficiency of liposomes was determined by centrifugation method according to the following protocol (Afrooz *et al.*, 2017). Aliquots (1 mL) of liposomal dispersions were subjected to centrifugation in a laboratory centrifuge (Mistral 1000 centrifuge, MSE, UK) at 15000 rpm for 10 min at 4°C. The clear supernatants were removed carefully to separate non-entrapped CV and from the entrapped (AA, CV) in the form of pellets. Then the pellet was disrupted using ethanol for releasing the entrapped drug. The absorbance of liposomal (AA, CV) was measured at λ max 589 nm (for CV) using UV spectrophotometer (M501, Spectronic Camspec, UK). Regarding the liposomal (DOX, AA, ALA), the method was the same except for the UV absorption wavelength which was 480 nm (for DOX). Then the encapsulation efficiency of CV and DOX was calculated using the slope obtained from the calibration curves through the following equation % EE= (encapsulated drug/ total drug) $\times$ 100%.

1.3.3 Storage Stability

Prepared liposomes were stored in glass vials in a dark place at 3-4°C and the changes in vesicle sizes and PDI were assessed over times. The plain liposomes and liposomal (AA) were measured at month 1, 2, 3, 4 and 5 while liposomal (DOX, AA, ALA) was assessed monthly for 11 months.

1.3.4 *In vitro* Drug Release Study of Liposomal (DOX, AA, ALA)

The *in vitro* drug release of doxorubicin from Liposomal (DOX, AA, ALA) was measured using the dialysis method. Briefly, a volume of 2 mL of freshly produced liposomes was added to a dialysis cassette (MWCO 3.5 kDa, 0.5-3 mL, ThermoFisher Scientific) and dialysed against 400 mL PBS, pH 7.4, at 37°C. The cassette was rotated at 100 rpm in a dissolution tester (DT 126 light Erweka, USA). At predetermined time intervals (0.08, 0.17, 0.25, 0.33, 0.50, 0.75, 1, 2, 3, 4, 6, 8, 12, 20, 25, 48, 72, 120, 144 and 168 h), 5 mL aliquots were withdrawn from inside the dissolution vessel. Afterwards, all samples were measured using the UV spectrometer at wavelength of 480 nm. The experiment was carried out in triplicates.

1.3.5 Transmission Electron Microscopy (TEM)

Liposomes were imaged with transmission electron microscopy (Hitachi H7000 transmission microscope, Japan) using negative staining technique. Specifically, a 3 μ L aliquot of liposome was fixed on carbon film mesh copper grid (400 mesh) (Agar Scientific Ltd, Essex, UK) using 50 μ L aqueous solution containing 2% (v/v) uranyl acetate stain (negative staining) for 45 sec. Excess sample was removed using the tip of a filter paper prior to application of the negative stain. Grids were left to dry prior their use. Images were acquired using a transmission electron microscope operated at an accelerating voltage of 75 kV.

1.4 Cell culture

For biology experiments, Dulbecco's Modified Eagle's medium (DMEM, 31053028), Roswell Park Memorial Institute Medium (RPMI 1640, 21875034) and Annexin V Apoptosis Detection Kit (88810272) were purchased from Thermo-Fisher Scientific (UK). Renal Epithelial Cell Growth Medium BulletKit™ (REGM, CC-3190) was purchased from Lonza Group Ltd (Switzerland). 2',7'-Dichlorofluorescein Diacetate (4091990), foetal bovine serum (FBS, F9665), trypsin- EDTA (T4174), L-glutamine (G7513)

and Sodium pyruvate (113-24-6) were all purchased from Sigma-Aldrich (Gillingham, UK). CellTiter 96® AQueous, MTS Reagent (MTS, G3582) was purchased from Promega (UK). Human breast adenocarcinoma cell lines MCF-7 (HTB-22™) and MDA-MB-231 (HTB-26™) were kindly gifted by Prof Alison Tyson-Capper, University of Newcastle, (UK). Primary Renal Proximal Tubule Epithelial Cells: Normal, Human (RPTEC, PCS-400-010) from ATCC was gifted by Dr Steven Darby, University of Sunderland, UK.

1.4.1 Cell Treatment Experimental Design

MCF-7 and MDA-MB-231 were treated with 6 formulations (Doxosome, Free DOX, Free (Dox, AA, ALA), Liposomal (Dox, AA, ALA), and Free (ALA, AA), in addition to the control group (media only with no drugs), While RPTEC was treated with (Doxosome, Free DOX, Free (Dox, AA, ALA) and Liposomal (Dox, AA, ALA)). Liposomal formulations used were chosen based on the optimal formulation obtained from the preliminary studies.

Briefly, MCF-7, MDA-MB-231 and RPTEC were seeded in 96-well plates (CLS3788, Sigma-Aldrich, UK) at a density of 10,000 cells per well. Then incubated overnight at 37°C before treatment. Afterwards cells were washed with fresh media then the cells were treated with different formulations (MCF-7 and MDA-MB-231 were treated with (Doxosome, Free DOX, Free (Dox, AA, ALA), Liposomal (Dox, AA, ALA), and Free (ALA, AA), while RPTEC was treated with Doxosome, Free DOX, Free (Dox, AA, ALA) and Liposomal (Dox, AA, ALA)). The untreated cells were used as a negative control. All cells were treated with different formulation groups of the same concentration range starting from 75 µg/mL with a serial dilution with ratio 2:1 and 5 dilution factors till 2.34 µg/mL. After 48 h exposure to given treatment cells were ready to be assayed.

1.4.2 Cell Proliferation Assay – MTS

MTS assay was carried out according to the manufacturer protocol (Promega MTS assay protocol) and applied to all cell lines. Briefly, cells were washed with the media twice then 20 µL of MTS solution was added to each well (treated/or untreated). In addition, a blank well and a negative control were used for background subtraction and calculation of cell viability. The 96-well plates were incubated for 1–4 hr in 5% CO₂ at 37 °C. Afterwards the plates were measured using the microplate reader (SpectraMax i3x, SoftMax Pro 7.0 software. Molecular Devices, UK) at 490 nm.

1.4.3 Measurement of Reactive Oxygen Species

The intensity of ROS production was evaluated using 2,7-dichlorofluorescein diacetate (DCFH-DA). After a 48 h incubation, treated cells were washed once with fresh warm media and twice with PBS 7.4 then incubated for 30 min with 100 µL DCFH-DA in a final concentration of 10 mM at 37 °C. Afterwards the cells were washed twice with PBS 7.4. DCF fluorescence intensity was measured using microplate reader (SpectraMax i3x, SoftMax Pro 7.0 software. Molecular Devices, UK) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

1.4.4 Apoptosis Assay

To detect the early stage of apoptosis, the PE-annexin V apoptosis detection Kit (Thermo-Fisher Scientific, UK) was utilised. MCF-7 and MDA-MB-231 cells were stained according to the manufacturer instructions. Briefly, the treated cells were incubated for 48h. The cells were washed twice with cold PBS (centrifuged at 1000xg for 5 min) and then suspended in 1x binding buffer at a concentration of

10⁶ cells/mL. Next, 100 µL of 10⁵ cells/mL was transferred to a separate tube and 5 µL of each of PE-annexin V and 7-AAD (7-amino-actinomycin D) were added. Afterwards, the solutions, were incubated in a dark place at room temperature for 15 minutes. Finally, 400 µL of 1x binding buffer was added to each solution, and they were subsequently analysed using Accuri C6 flow cytometer (BD Pharmingen, UK) within one hour.

1.5 Statistical Analysis

Data analysis was conducted using GraphPad Prism 9 software (CA, USA). Each experiment was carried out in triplicates (n=3) and each measurement was taken in triplicate (n=3), and the standard deviation (SD) for each experiment was calculated. All data points plotted on graphs represent the mean of three independent experiments $\pm$ SD, unless otherwise stated. A p value of less than 0.05 ($p < 0.05$) was considered statistically significant. The statistical significance of data was evaluated using Student's *t*-test, one-way ANOVA, and two-way ANOVA for multiple comparisons. One-way ANOVA was specifically used to compare the control group against different treatment groups individually. Since the objective of the study was to assess significant differences between the control and each treatment group individually, rather than comparing all groups to each other. The use of one-way ANOVA ensured the robustness of the analysis by minimizing the risk of Type I error while addressing the specific hypothesis of control vs treatment comparisons.

2 Results and discussion

2.1 Characterisation of Plain Liposomes

Ideal composition of plain vesicles was selected based on a preliminary study to achieve a suitable incorporation of the drug compounds into the formulation. The results were explained through the principle of DLS technique. The neutral phospholipid, DSPC, has been chosen as a model membrane component for liposomal formulations since it mimics the biological membranes in many aspects, being one of their most abundant constituents (Shafaa, 2020). DSPC has showed a high level of stability, drug retention and decreased the drug leakage. This is because of its long acyl group that exerts high transition temperature and its cylindrical geometric structure bearing small-size head group and liposomes formulated with lipids of high transition temperatures have showed higher stability than those with lower transition temperatures (Anderson and Omri, 2004; Nakhaei et al., 2021; Ghosh et al., 2022). In this study, total flow rate (TFR) of 12 mL/min was chosen as high TFR lead to smaller vesicles, please add citation.

Three plain liposomal formulations were selected from the initial six formulations for further studies based on preliminary results (Table 1). These three formulations exhibited the lowest polydispersity index (PDI) and the smallest vesicle size, mostly ranging between 50–150 nm (Table 2), which is considered optimal for in-vivo performance (Beltran-Gracia et al., 2019). The remaining three formulations were excluded due to their larger mean diameters, as observed in the DSPC:CHOL/microfluidics flow rate ratios of 1:1/1:1, 1:1/2:1, and 2:1/1:1, with vesicle sizes of 104.6 ± 14.0 nm, 208.2 ± 48.0 nm, and 103.6 ± 2.0 nm, respectively. It was also observed that the flow rate ratio (FRR) is inversely proportional with vesicle size, where the higher the FRR the smaller the vesicle size. On the other hands, increasing lipid concentration has resulted in a decrease in the vesicle size

and consequently, the PDI. Moreover, cholesterol was also found to influence liposomes' size where increasing cholesterol concentration increases size (Olusanya *et al.*, 2018).

Previous research reported the effect of different parameters on different liposomal formulations including DSPC: Cholesterol ratio. A recent study reported that the increase of initial lipid concentrations of DSPC (from 0.3 to 10 mg/mL) decreased the liposome size irrespective of the lipid selected. It was also reported that the increment in FRR of the solvent (aqueous: non-aqueous) influenced the decrease in the liposomal size. The FRR of 1:1 produced larger liposomes 100–200 nm depending on the initial lipid concentration, while FRR of 3:1 produced liposomes in a smaller size range (50–60 nm) and FRR of 5:1 produced size around 50 nm with more heterogeneous populations (Forbes *et al.*, 2019). Another study supporting the findings, stated that the increase in FRR of the solvent (aqueous: non-aqueous) phases from 1:1 to 5:1 caused a significant reduction in the liposomes size (Kastner *et al.*, 2014). This was explained by the variation in the alcohol concentration present for the given FRR, and as FRR decreases, there is an increase in the quantity of alcohol discharged into the stream. Initially, upon the interaction of lipids in alcohol with the aqueous stream, liposomes tend to self-assemble at the interface. The continuous mixing eventually reduces the alcohol concentration in the liposomes, resulting in re-assembly. However, a rise in FRR reduces the overall alcohol concentration, thereby limiting the assembly/re-assembly cycle of the liposomes that are exposed to the fluctuating increments and decrements of alcohol concentration (Jahn *et al.*, 2007; Zizzari *et al.*, 2017). Consistently with the previously mentioned studies, the formulations prepared in our research at higher FRR (2:1 and 5:1) demonstrated a significant decline in vesicle size when compared to that at FRR (1:1) with a statistical significance of ($p < 0.05$). Finally, the physiochemical characterisation led to the above conclusion, which will be explained thoroughly in the below **(section 2.2)**. Hence, the lipid type (DSPC), lipid concentration, DSPC:cholesterol ratio, and aqueous:non-aqueous flow rate ratios were selected as the key parameters for this study. As these choices were driven by their significant role in achieving optimal vesicle size, enhancing colloidal stability, and improving drug retention, ensuring the formulation's suitability for further development and applications.

The zeta potential obtained indicates the stability of the formulation. The zeta potential is frequently used to determine the stability of colloidal systems. Typically, zeta potentials between +30 mV and -30 mV are considered stable (Laouini *et al.*, 2012). Zeta potential values for all formulations were slightly negative with no statistical difference, as expected since the lipid used in the study is zwitterionic under the tested pH (**Table 2**). Although, in the present study, liposomes prepared with DSPC, which is a neutral lipid, possessed a slight negative charge. This finding could be due to the lipid and/or cholesterol oxidation where it was found in previous research that lipid or cholesterol oxidation increases the negative value of zeta potential (Mosca *et al.*, 2011). Previous investigations have reported that the introduction of cholesterol resulted in an augmentation of the negative zeta potential of liposomes (Liu *et al.*, 2000). The presence of negative charge benefits the formulation in terms of decreasing the attraction forces between the liposomal particles, as overall zeta potential was low, charge stabilization is not anticipated much for colloidal stabilization (Yang *et al.*, 2007).

TEM were obtained for the selected formulations with ratios 1:1/ 5:1, 2:1/ 2:1 and 2:1/ 5:1 to confirm the size and the shape of the liposomes. All liposomes were found to be spherical, well dispersed, and unilamellar in their structures (Figure 1A). The findings confirm the DLS measurements, where the vesicle size measured by TEM corresponds with the average vesicle size obtained via DLS.

Insert Figure 1 and Table 2

2.2 Characterisation of liposomal formulations with doxorubicin, DOX, ascorbic acid, AA and alpha lipoic acid, ALA

Liposomal (DOX.AA. ALA) was prepared in three different formulation ratios as previously mentioned. The size analysis showed that the formulation (DSPC: cholesterol/ PBS buffer: ethanol microfluidics flow rate ratio) of (1:1/ 5:1) had a significant ($p < 0.05$) smaller sized vesicles 79.0 ± 0.3 nm compared to liposomes prepared with other ratios (Table 2). The PDI of the three liposomal formulations had values less than 0.1, which indicates uniformity and homogeneity, where values between 0.1 and 0.25 display more uniformity, mono-dispersity and physical stability (Sabeti *et al.*, 2014). Although DOX contains an amino group with a positive charge, the zeta potential was slightly negative noting that the lipid (DSPC) used in the study is a neutral phospholipid. ALA may have an influence on the surface charge, where liposomal dapoxetine co-loaded with 15% and 25% of ALA decreased the ZP to 20 mV and to less than 1mV, respectively (El-Say e *t al.*, 2019; Gu *et al.*, 2016), this could be attributed to the adsorption of anionic carboxyl group of ALA on the particle surface leading to neutralisation of the positive surface charge of dapoxetine. A recent study assessing the effect of AA as a life extender and stabiliser in liposomes has reported that negative ZP of a blank liposome made with neutral lipid increased from - 4.5 mV to - 7.3 mV when the neutral lipid liposomes was loaded with AA. The increase of the negative value influenced by AA can be a result of pKa (4.04) of AA, Where the molecules ionize and release hydrogen atoms of AA, hence the increase in the negative ZP value (Favarin *et al.*, 2023).

Entrapment efficiency of the liposomal (DOX.AA. ALA) was consistent with a preliminary result obtained from using crystal violet, CV, as a hydrophilic mocked drug. Liposomal (CV. AA) with ratio of (1:1/ 5:1) (lipid: cholesterol/ PBS: ethanol) showed the highest encapsulation of CV ($76.0 \pm 2.1\%$) while the least was for (2:1/2:1) and (2:1/ 5:1) (Table 2). The result indicated that the increase in cholesterol concentration increased the liposome size which consequently increases the drug encapsulation efficiency. Thus, the liposomes with ratio of (2:1/2:1) and (2:1/5:1) showed lesser CV encapsulation since they comparatively had less cholesterol and DSPC concentration is twice as much of cholesterol. While an increased level of cholesterol was reported to insert itself into the bilayer membrane, preventing the drug leakage (Shirsand *et al.*, 2012). The liposomal formulation with ratio (1:1/ 5:1) had the highest DOX encapsulation efficiency of $97.0 \pm 0.6\%$ with high significance level ($p < 0.001$) when compared to ratios of (2:1/2:1) and (2:1/5:1) with DOX entrapment of 13.0 ± 1.0 and $52.0 \pm 3.0\%$, respectively (Table 2). Thus, the (1:1/ 5:1) formulation ratio was chosen as the main novel formulation ratio for further assessments (sections 2.2.1, 3.2, 3.4 and 3.5).

2.2.1 DOX release profile in liposomal (DOX. AA. ALA)

The release of DOX from liposomal (DOX.AA.ALA) followed a biphasic manner, starting with a burst phase (~40% within 6h at 37°C under sink conditions in PBS 7.4), followed by a sustained release (~70% at 25h, 80% at 48h, and 87% at 72h), with complete release (98.1%) at 168h (Figure 1B). The biphasic release likely results from the detachment of surface-adsorbed DOX during the burst phase, followed by slower release from the inner lamellae (Panwar *et al.*, 2010; Roy *et al.*, 2016). Moreover, It was reported that the release of DOX from the commercial liposomes, Doxil, take action in multiple stages: first the solid crystals are dissolved, secondly the free soluble drug is protonated then deprotonated and finally the neutral drug is transported passively across the bilayer. (Russell *et al.*,

2018). Therefore, another possible reason is that DOX was not completely crystallised when entrapped inside the liposomes leading to higher diffusion rate upon heating condition. Previous research has reported that unbundled DOX had 5 times faster release rate when compared to bundled form (Swenson *et al.*, 2001).

Similar studies reported DOX release of 70–80% at 72h from cationic and anionic liposomes (Nie *et al.*, 2012) and ~76% at 52h from liposomal DOX, compared to 89–93% release within 2h for free DOX (Ghaferi *et al.*, 2022). Further, PEGylated DOX liposomes showed significantly slower and/or delayed release when compared to the conventional DOX liposomes and the free DOX (Ghaferi *et al.*, 2022). Notably, our non-PEGylated formulation demonstrated acceptable drug retention and release times. Finally, a study reported that palmitoyl ascorbate and doxorubicin co-encapsulated liposomes had slower drug release when compared to DOX coloaded liposomes and free DOX and suggesting that the anti-oxidant palmitoyl ascorbate (ascorbic acid derivative) had an influence in increasing the drug retention time within the liposomes (Yang *et al.*, 2017). Antioxidants in our formulation likely reduced lipid hydrolysis and oxidation, improved stability, and enhanced encapsulation efficiency, contributing to improved drug release and retention (Li *et al.*, 2016; Yang *et al.*, 2017; El Adawy *et al.*, 2018; García Esteban *et al.*, 2018; Ling *et al.*, 2019).

2.3 Storage Stability of liposomes

2.3.1 Effect of Storage on Plain Liposome versus Liposomal (AA)

The stability of drug-loaded liposomes is vital for storage and clinical application. The change of vesicle size and PDI were used for evaluating storage stability. The size and PDI were measured directly when they came out of the microfluidic system (ID system) then the measurements were repeated monthly for a duration of 5 months (M) in storage temperature of 4°C. Plain liposomes made of DSPC and Chol of ratio 1:1 and FRR of 5:1 obtained size and PDI of 71.6 ± 5.1 nm and 0.2 ± 0.03 , respectively, when measured directly after preparation, and increased to 519 ± 197 nm and 0.6 ± 0.15 , respectively, after 5 months of storage. The size and PDI of plain liposomes increased upon increasing the storage times, where one-way ANOVA showed a high level of significance ($p < 0.0001$) between the results obtained directly from the system and all the measured groups throughout the 5 months (M1 to M5) (Table 3).

On the other hand, it was observed that the same liposomes composition coloaded with AA showed less increase in size and PDI, where one way ANOVA showed a statistical difference in size and PDI between liposomes measured direct from the system and 5 months of storage (M1- M5) (Table 4). However, it's noteworthy to mention that the size increase of liposomes coloaded with AA during the 5 months remained small. The mean size of particles measured at M5 were 98.0 ± 2.6 nm, which is still an acceptable size range for enhanced permeability and retention effect for the accumulation and retention of liposomes in the tumour interstitium (Olusanya *et al.*, 2018). There was a change in the PDI value of the liposomal (AA) upon storage, though the results obtained from M1 to M5 were below 0.4 and sample was still considered stable as the recommended PDI for high stability was reported to be below 0.3 (Danaei *et al.*, 2018). Moreover, there was a high level of significant difference in size and PDI at ($p < 0.0001$ based on T-test) between the M5 of liposomal (AA) and plain liposomes. The results indicated that the presence of AA stabilised the formulation, preserved the vesicle size for 5 months of

storage, prevented/reduced oxidation, reduced fusion, and aggregation when compared to the liposomes that had no AA. The significant increase in size for plain liposomes could be explained by formulation instability caused by hydrolytic degradation and oxidative degradations (Boggula *et al.*, 2022). Hydrolytic degradation can occur due to Hydrolysis of ester bonds forming lysophospholipids then phosphoglycerols (Rideau *et al.*, 2018; Siriwardane *et al.*, 2020), while oxidative degradation can be a result of oxidation of the unsaturated acyl chains due to the presence of the free radicals/ ROS in the fatty acid (Anderson and Omri, 2004; Çağdaş *et al.*, 2014; Nakhaei *et al.*, 2021).

Insert Tables 3 and 4

The use of antioxidants was reported to increase the stability of liposomes and reduce lipid oxidation (Çağdaş *et al.*, 2014). For example, the use of liposomes coloaded with astaxanthin as antioxidant strongly inhibited the leakage and aggregations at storage. It was also reported that encapsulation enhanced the antioxidant activity of astaxanthin (Pan *et al.*, 2018). Lipka *et al.* reported that the encapsulation of AA with liposomal epirubicin, composed of HSPC/Chol/DSPE-PEG 2000 increased the stability of the formulation during storage and prevented lipid hydrolysis. Encapsulation of 300 mM of AA in epirubicin liposomes increased the stability and the shelf-life of the formulation when stored at 4 and 24°C. There was no significant difference in stability regarding size and PDI upon 1 year of storage at 4°C, however a marginal drug release was observed over 6 months storage at 24°C (Lipka *et al.*, 2013). Another study reported that the co-encapsulation of antioxidants such as AA and folic acid had a good stability effect on liposomes made of soybean phosphatidylcholine during the long-term storage at relatively low temperature. The liposomes were stable with no significant size increase when stored at 4°C and a slight increase in size when stored at 25°C for 40 days, indicating that the antioxidant prevented the fusion and aggregation of particles upon storage (Jiao *et al.*, 2019). Recent research studying the effect of AA as a liposome stabiliser and shelf-life extender reported that liposomes made of neutral lipid and loaded with AA had a higher stability when compared to the plain liposomes that had no AA. The liposomes coloaded with AA prevented lipid oxidation by neutralising free radicals, induced more negative ZP which consequently increased lipid stability and stabilised the formulation for 25 days where vesicle size (160 nm), PDI (0.23) and ZP remained unchanged. While liposomes with no AA were only stable for 10 days and there was a significant change in vesicle size (above 300 nm), PDI (above 0.4) (Favarin *et al.*, 2023). The effect of AA can be explained by the fact that AA is degrading to maintain stability of liposomes. As AA concentration was found to be gradually decreasing from the initial reported concentration (1 mg/ mL) to (0.06 mg/mL) at 20 days. Another study supporting the mechanism of stabilization by AA reported that antioxidant activity of AA is produced from the displacement of ascorbic acid to its oxidized form, dehydroascorbic acid, that combats the free radicals produced by the formulation or liposomes drug content. Upon storage, the balance between ascorbic acid and dehydroascorbic acid is displaced to the right side, rendering ascorbic acid to dehydroascorbic acid. Hence, the loss of the AA capacity to scavenge free radicals (García Esteban *et al.*, 2018).

2.3.2 Effect of storage on liposomal (DOX. AA. ALA)

The stability (size and PDI) of liposomal (DOX. AA. ALA) of the selected lipid and FRR ratios (1:1/ 5:1) was determined directly after preparation using the DLS, then the mean diameter measurements were repeated monthly for a total duration of 11 months (M) at a storage temperature of 4°C. It was observed that the liposomal (DOX. AA. ALA) showed a high level of stability upon storage at 4°C. One way ANOVA test showed no statistical difference at ($p > 0.05$) between the size obtained directly from the system (fresh samples) and the size obtained from M1 to M6, where vesicle size measured directly after preparation and at M6 were 79.0 ± 0.3 and 82.0 ± 1.1 nm, respectively. Then there was a statistical difference ($p < 0.05$) between the size measured after preparation and between M7 to M11 (Table 5). The vesicle sizes till M11 didn't exceed 150 nm which is still an acceptable size range for good internalization and retention in the tumour interstitium (Olusanya *et al.*, 2018). Regarding the PDI, one way ANOVA showed no statistical difference ($p > 0.05$) between PDI measured directly from the system (0.1 ± 0.01) and between (M1 to M5). All PDI results (M1 to M11) were below 0.3, indicating that liposomes were highly monodispersed (Table 5) (Refai *et al.*, 2017; Danaei *et al.*, 2018).

Insert Table 5

3 Cytotoxicity and ROS Measurements in Breast Cancer Model

3.1 Cytotoxicity of Free DOX and Its Influence on ROS Production in Breast Cancer Model

The cytotoxic profile of free DOX towards selected breast cancer cell lines over 24 h treatment was found to be highly potent. Two mechanisms have been suggested for DOX anti-cancer effect. First is intercalation into DNA and disruption of topoisomerase-II-mediated DNA repair and second is the generation of free radicals leading to oxidative stress, damage to cellular membranes, DNA, and proteins. Topoisomerase II is responsible for the survival and division of the cancer cells; hence, its poisoning leading to DNA damage is vital as cancer cells are more vulnerable to DNA breakage than healthy cells (Thorn *et al.*, 2011; Jawad *et al.*, 2019; van der Zanden *et al.*, 2021; Peter *et al.*, 2022). It was observed that free DOX at low concentrations (2.3, 4.7 µg/mL) exerted remarkable cell inhibition on MCF-7 and MDA-MB-231 when compared to higher concentrations (9.4, 18.7, 37.5 and 75 µg/mL) specially for MCF-7, which had a higher cell inhibition than MDA-MB-231 of same treatment concentration (Figure 2).

Insert Figure 2

One way ANOVA showed a statistical difference ($p < 0.05$) between the control group and all DOX concentrations for both cell lines. It was also observed that high concentration of DOX showed less cell inhibition when compared to the low concentration. This could be attributed to the fact that some anti-cancer drugs exert a hormetic dose response, where hormesis is the phenomenon of biphasic dose response in which small doses of an intervention such as radiation, drugs or immune reactants show stimulatory effects on targets, while high doses show inhibitory effects, thus posing significant challenges for decision-making in therapies of cancer (Bao *et al.*, 2015; Tang *et al.*, 2023). It was reported that anticancer drugs such as cisplatin, 5- fluorouracil, doxorubicin, mitomycin, etoposide, irinotecan, docetaxel, paclitaxel and gemcitabine had a hormetic response in cancer cells (Yoshimasu *et al.*, 2015). However, further investigations are needed to understand the biphasic response of DOX. Our results were comparable with those reported in recent literature using the same cancer cell lines and same cell viability protocols, where concentration of DOX between 5, 2.7, and 0.5 µg/mL had a higher cytotoxic effect and growth inhibition on MCF-7 and MDA-MB-231 cells (Arif *et al.*, 2013; Pilco-Ferreto and Calaf, 2016; Wen *et al.*, 2018). These findings suggest that the biphasic dose response of DOX may have significant clinical implications for cancer therapy. Optimising the dosage to achieve a balance between efficacy and minimising side effects such as cardiotoxicity could enhance the therapeutic index of DOX in clinical settings.

Regarding the ROS measurements, it was observed that all free DOX concentrations significantly induced very high percentage of ROS production (above 100%) in both cell lines and especially the MCF-7 when compared to the untreated control cells (which is expressed as 100%). It was also observed that there was a direct relation between cell growth inhibition and the ROS production level, and concentrations that had higher cell inhibition exerted higher intercellular ROS production and vice versa. The most common adverse effects of DOX are cardiotoxicity, hepatic damages, and nephrotoxicity. Many studies have found that DOX induced cell toxicities are mainly caused by DOX induced oxidative stress, resulting in excessive ROS generation (Su *et al.*, 2015; He *et al.*, 2020). DOX

induces the production of ROS through two major pathways: iron dependant non-enzymatic pathway and enzymatic mitochondrial respiratory chain pathway (Wang *et al.*, 2015; Mobaraki *et al.*, 2017). The latter is considered as the main cause of organ toxicity (Kim *et al.*, 2006). Mitochondria are considered the main intracellular source of ROS where ROS are produced at very low levels during mitochondrial respiration under normal physiological conditions (Wang *et al.*, 2015; Mobaraki *et al.*, 2017). The formation of ROS takes place when unpaired electrons escape the electron transport chain and react with molecular oxygen. Under normal physiological conditions there's a balance between the production and the elimination of ROS and disturbance to balance of the redox reaction will lead to oxidative stress. This could either happen due to an increase in ROS generation, decrease in antioxidant capacity, or a combination of both (Wang *et al.*, 2015; Mobaraki *et al.*, 2017). In the DOX case, the reaction takes place when a lone pair of electrons from DOX is lost during the formation of doxorubicin semiquinone by the aid of reduced flavoenzyme such as NADPH-cytochrome P450 reductase. (Kim *et al.*, 2006; Thorn *et al.*, 2011). Ultimately inducing the membrane lipid peroxidation, DNA and membrane damages (Thorn *et al.*, 2011; Afsar *et al.*, 2020). In relation to our study, recent research reported that DOX induced cell viability inhibition in H9c2 myoblasts (cell model used as an alternative for cardiomyocytes) in all concentrations used (0.1–20 μ M), however a significant cytotoxicity was observed only in the presence of 5.4 and 10.8 μ g/mL of DOX which is close to the concentration range in our research that showed the highest cell inhibition and ROS production. In addition, cells treated with DOX showed a reduced expression of superoxide dismutase (SOD2) (Sirangelo *et al.*, 2022). SOD2 is a mitochondrial matrix protein that plays a critical role in protecting the cardiovascular system through its antioxidant function. Deficiency in SOD2 expression results in vascular endothelial dysfunction, hence the cardiotoxicity (Tseng *et al.*, 2017). Liang *et al.* (2025) demonstrated that co-delivery of curcumin (which has anti-inflammatory effect) with doxorubicin in a modified liposomal form reduced doxorubicin cardiotoxicity, in vivo experiment, when compared with free DOX suggesting that their liposomes have a potential to be a safe therapeutic possibility for cancer treatment as it showed a significant inhibitory effect on tumour growth in vivo while demonstrating a positive toxicity profile.

3.2 Cytotoxicity of Liposomal (ALA.AA), Free (ALA.AA) and Their Influence on ROS Production in Breast Cancer Model

The effect of liposomal (ALA.AA), free (ALA.AA) and their influence on ROS production on breast cancer model were assessed as a proof of concept, to test whether the antioxidants combination either in free form or in liposomal form would exert an effect on MCF-7 and MDA-MB-231 after 24 h incubation or not. The tested concentrations of liposomal (ALA.AA) and free (ALA.AA) (0.2, 0.5, 0.9, 1.9, 3.8 and 7.5 mg/mL) were exactly of the same ratio as used in liposomal (DOX.AA.ALA) of different concentrations. Low concentrations used in our study of either liposomal (ALA.AA) or free (ALA.AA) had a statistical significance on cell viability on both cell lines compared to the control group, where low concentrations (0.2 and 0.5 mg/mL) used in our study of either liposomal (ALA.AA) or free (ALA.AA) had shown significantly better cell viability and lower ROS production on both cell lines compared to the control group (data not shown).

AA is acknowledged for its contradictory activity, as it acts as an antioxidant at low concentrations, while at higher concentrations, it acts as a pro-oxidant. The pro-oxidant effect of AA induces cell inhibition by

producing ROS in cancerous cells and restricting ATP production through the glycolytic pathway (Maekawa *et al.*, 2022). It was observed that AA can selectively target the tumour cells without causing any damages to the normal surrounding cells. High concentrations of AA in millimolar range can enter the cancer cells generating ascorbate radicals and hydrogen peroxide inside the cancer cells that lead to cellular component damage and cell death (Chen *et al.*, 2008; Takemura *et al.*, 2010; Baek *et al.*, 2017). Otto Warburg and his colleagues observed that cancer cells exhibit high glucose consumption through the glycolysis pathway when oxygen is scarce. This metabolic state is commonly named as the 'Warburg Effect' (DeBerardinis and Chandel, 2016). The upregulation of glucose transporter-1 (GLUT1) genes, facilitated by KRAS or BRAF gene mutations observed in cancer cells, is responsible for promoting the Warburg Effect (Yun *et al.*, 2009). Under hypoxic conditions, cancer cells remain viable through increased glycolysis, which is mediated by the selective upregulation of GLUT1. As such, targeting this mechanism of glucose consumption may prove to be a promising therapeutic approach for the treatment of cancer (Barbosa and Martel, 2020). Additionally, upregulation of GLUT1 allows cancer cells to undergo glycolysis at high rates approximately 200 times faster than normal cells (Zhang, 2015). AA undergoes oxidation and transforms into dehydroascorbate (DHA) while inside the body (Yun *et al.*, 2015). Through an accelerated glycolytic pathway that results in an increased level of glucose transporter-1 (GLUT1) expression, DHA finds a higher absorption rate (Maekawa *et al.*, 2022). Considering the structural resemblance between glucose and DHA, upregulated GLUT1 in cancer cells permits a substantial amount of DHA to diffuse into the cells (Ngo *et al.*, 2019). As soon as DHA enters the cancer cells, it is reduced back to AA through the action of glutathione (GSH) and nicotinamide adenine dinucleotide phosphate (NADPH) (Yun *et al.*, 2015). The reduction process of DHA leads to the conversion of NADPH and GSH to oxidize nicotinamide adenine dinucleotide phosphate (NADP⁺) and glutathione disulphide (GSSG), which gives rise to the production of ROS inside the cancer cells (Figure 3) (Yun *et al.*, 2015; Tu *et al.*, 2017).

Insert Figure 3

The accumulation of ROS such as hydrogen peroxide in cancer cells, coupled with the low activity of catalase in cancer cell, can lead to DNA damage. This DNA damage triggers the activation of Poly ADP-ribose polymerase (PARP), which depletes nicotinamide adenine dinucleotide (NAD⁺), an important cofactor in the conversion of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to nicotinamide. This leads to less production of ATP, hence, the cellular dysfunction and death through a process of apoptosis (Yun *et al.*, 2015; Maekawa *et al.*, 2022). Its noteworthy to mention that the previously explained mechanism is selective to cancer cells while having no effect of the normal surrounding cells. Therefore, cancer cells are sensitive to ROS and the effects of ascorbic acid due to the high expression of GLUT1. In addition, normal cells have sufficient catalase to disable hydrogen peroxide and ROS while cancer cells have less catalase activity rendering it more vulnerable and susceptible to AA anticancer effect (Lee *et al.*, 2019; Reang *et al.*, 2021).

Previous research reported that AA in millimolar concentration (1–4 mM) acted as a prooxidant and significantly reduced the cell viability of HCT116 and MCF7 cell lines with no effect on normal cells (Ganash, 2021). A recent study reported that AA had an efficient anti-cancer activity in human colorectal cancer cell when used in concentration from 10 μ M to 2 mM. On contrary, AA exhibited a biphasic effect in cancer cells, whereas concentration >1 mM showed high growth inhibition and apoptosis effect while low concentrations <10 μ M induced cell proliferation as a result of hormetic dose response as AA cannot generate sufficient ROS to kill cancer cells (Cho *et al.*, 2018). On the other hand, ALA anticancer effect was referred to its antioxidant properties. ALA was found to have a high percentage of cell inhibition on LNCaP cell line (human prostate cancer cell line) when used in concentration range between 250 μ M and 2 mM. ALA acts as a free radical scavenger in the prostate cell lines. The level of oxidative stress was lower in the prostate cancer cells that were treated with ALA than in the untreated cancer cells. ALA promotes the expression of Ref-1 protein in prostate cancer cells suggesting that ALA may increase DNA damage of cancer cells under pathologic condition, where Ref-1 protein has a crucial role in the transcriptional response to oxidative stress and in DNA base excision repair (Choi *et al.*, 2009). Another study reported that incubation of MDA-MB-231 with ALA at concentration range of 250 to 1000 μ M for 48 h led to a significant decrease in cell proliferation ($p < 0.05$) (Na *et al.*, 2009). Finally, previous research supporting our findings reported that, not only ALA was toxic to tumour cells at mM concentrations but also synergised the AA anti-cancer effect when used in a combination. No sufficient explanation was provided for the reason behind the synergism however it was suggested that the combination of AA and ALA may modify cellular oxidation-reduction status in a manner that enhances H₂O₂ toxicity or AA may have been recycled through the ALA in redox reactions (Figure 4) (Casciari *et al.*, 2001). Lal *et al.* (2023) reported in a study, but not on liposomes, that ALA considerably protected rat hippocampal against DOX-induced memory impairment and it also stopped DOX-induced oxidative and inflammatory effects via NRF-2/HO-1 levels' upregulation, hence ALA showed neuroprotective effects. Finally, suggested synergy between AA and ALA in liposomal formulations may allow for more effective treatment regimens in cancer therapy, improving the specificity of antioxidants in targeting tumour cells while minimising systemic exposure and associated side effects.

3.3 Cytotoxicity of Liposomal (DOX.ALA.AA), Doxosome and Free (DOX.ALA.AA) and Their Influences on ROS Production in Breast Cancer Models

The cytotoxicity of the liposomal (DOX.ALA.AA) were tested against MCF-7 and MDA-MB 231 cell lines for 24 h. Multiple concentrations were tested against the cells, where concentrations of DOX were (2.3, 4.7, 9.4, 18.7, 37.5 and 75 µg/mL) and ALA and AA were (0.2, 0.5, 0.9, 1.9, 3.8 and 7.5 mg/mL). MTS assay showed that low concentrations of liposomal (DOX.ALA.AA) exerted significant cell growth inhibition on both cell lines ($p < 0.05$) when compared to the control group (non-treated cells). It was observed that adding 2.3, 4.7 and 9.4 µg/mL of DOX had led to higher cytotoxicity, with cell viability of 14.93, 53.30 and 58.80% for MCF-7 and 39.14, 39.01 and 67.80% for MDA-MB 231 cell lines, respectively (Figure 4A, C). Both cell lines treated with liposomal (DOX.ALA.AA) showed a significant decreased ROS production in all concentrations used when compared to the control group (Figure 4B, D). Free (DOX.ALA.AA) showed significant cytotoxic effect ($p < 0.05$) for low concentrations when compared to the control group for both cell lines. Cell viability % of treated DOX concentrations 2.3 and 4.7 µg/mL were 46.7 and 39.7% for MCF-7, respectively, While the cell viability of the same concentrations was 62.8 and 70.6% for MDA-MB 231, respectively. Regarding the ROS production, all concentrations used of free (DOX.ALA.AA) reduced the ROS production when compared to the control group for both cell lines. It was noteworthy to mention that the levels of ROS induced by liposomal (DOX.ALA.AA) were less than the ROS produced by free (DOX.ALA.AA) on both cell lines. On the other hand, Doxosome cytotoxicity and ROS production in both cell lines adapted a similar manner to the results obtained from the liposomal (DOX.ALA.AA) (Figure 5A). However, the induced ROS level, was observed to be higher in both cell lines than the ROS level produced by the liposomal (DOX.ALA.AA) (Figure 5B).

Our results showed that our liposomal formulation exceeded the efficiency of the free (DOX.ALA.AA) and Doxosome. Noting that Doxosome, according to the manufacturer, is a PEGylated liposomal DOX, which is a replica from the commercial Doxil® in size, lipid composition and the amount of the encapsulated drug. Low concentrations of liposomal (DOX.ALA.AA) at 2.3 and 4.7 µg/mL showed the highest level of cell inhibition and the lowest ROS production on both cell lines when compared to free (DOX.ALA.AA) and Doxosome. The results can be attributed to synergistic effect of the pro-oxidant and antioxidant effects of AA and ALA. Previous research reported that a combination of natural antioxidants such as AA and ALA can synergise the anti-cancer effect of chemotherapies (Yang *et al.*, 2019; Attia *et al.*, 2020; Maekawa *et al.*, 2022). It was reported that, AA can sensitise different cancer cells to common therapy strategies, where the addition of AA to cisplatin as a combinational therapy enhanced the *in vitro* anticancer effect of cisplatin with five times less than IC₅₀ doses in synergistic manner (Ghavami and Sardari, 2020). Other studies have also revealed that combination of AA with chemotherapies such as 5-fluorouracil, sodium d-ascorbate, cyclophosphamide, paclitaxel, arsenic trioxide, vincristine, doxorubicin can sensitise the cancer cells to the anticancer drugs, potentiate the anti-cancer effect and enhance the success rate of chemo/radiotherapy (Wilson *et al.*, 2014; Longchar and Prasad, 2015; Singh *et al.*, 2018).

Insert Figures 4 and 5

Numerous studies have suggested that antioxidants, such as AA and ALA, play vital roles in enhancing and synergising the cytotoxic effects of antineoplastic agents while simultaneously protect healthy body tissue. This subsequently results in a decrease in chemotherapy side effects and an increase in patient survival rates (Attia *et al.*, 2020). A recent study revealed that administering AA or cisplatin alone to Dalton's lymphoma ascites-infected mice resulted in an increase in the host's mean survival time, around 79% and 122%, respectively. However, combining AA and cisplatin increased survival rates by about 142%, accompanied by a decrease in tumour size. The study reported that AA synergised the therapeutic effectiveness of cisplatin by reducing glutathione and catalase activity while elevating lipid peroxidation levels in Dalton's lymphoma tumour cells. Furthermore, AA played a protective role against cisplatin-induced tissue toxicities, where histological analysis showed that cisplatin treated mice had a destruction of the renal tubular cells close to grade 2 damages with elevated level serum urea and creatinine and marked elevation of hepato-cellular markers ALT, ALP, and AST, indicating hepato-cellular injury. However, administration of AA reduced the destruction of renal tubular cells with lesser glomerular damage, hence, the reduced nephrotoxicity. Additionally, pre-treatment with AA significantly attenuated the elevated hepato-cellular markers suggesting its hepatoprotective effect (Longchar and Prasad, 2015).

ALA was also found to significantly inhibit cell growth and proliferation in neuroblastoma and breast cancer cell line (Feuerecker *et al.*, 2012). ALA at a concentration of 250 $\mu\text{mol/L}$ or higher for 48 h led to a significant decrease in cell proliferation and induction of apoptosis ($p < 0.05$) in MDA-MB-231 cell line (Na *et al.*, 2009). Based on our data and previous research, the combination of AA and ALA had a significant cytotoxic effect especially when loaded onto liposomal carriers where they exerted a higher percentage of cell inhibition. In the line of our findings, co-encapsulation of AA on paclitaxel nanoparticles showed a significant tumour growth suppression in B16F10-bearing mice and in elimination of cancer cells that have metastasised in the lungs than either AA or paclitaxel alone. In addition, the nanocarrier formulation showed an increase in cellular permeability and uptake of drugs in B16F10 cells (Zhou *et al.*, 2017).

Another study supporting our findings reported that, encapsulation of ascorbyl palmitate with DOX significantly decreased the cell viability of MCF-7 cells; HepG2 cells (human liver hepatocellular carcinoma cells) and A549 cells (human lung carcinoma cells) when compared with single DOX or ascorbyl palmitate liposomes. Moreover, the liposomes co-loaded with DOX and ascorbyl palmitate had a higher DOX internalisation inside the tumour cells with higher cytotoxic level. The uptake of liposomes co-loaded with DOX and ascorbyl palmitate were 2.5-folds greater than liposomal DOX and 5.4-folds greater than free DOX solution. The same formulation was also shown to reduce the tumour size and weight in MCF-7 bearing mice, where liposomes co-loaded with DOX and ascorbyl palmitate treated group showed lighter tumour weight 2.77 ± 0.53 g than saline group (6.55 ± 0.57 g) and liposomal DOX group (4.33 ± 1.04 g) (Yang *et al.*, 2017). Diacetyldichlorofluorescein diacetate assay revealed that our formulation liposomal (DOX.AA.ALA) induced the least ROS% in the treated cell lines when compared to Doxosome and free (DOX.ALA.AA). Hence, liposomes improved the antioxidant effect of the

encapsulated AA and ALA, reduced DOX induced toxicity by ROS production with better protection of normal tissues from chemotherapy induced damage and ameliorate toxicity level (Singh *et al.*, 2018). A prospective study that was randomised, double-blinded, and placebo-controlled evaluated the protective effect of ALA in patients with stage II and III breast cancer who were subjected to paclitaxel-induced peripheral sensory neuropathy and doxorubicin-related cardiotoxicity. The study found that administering 600 mg of ALA daily as an adjunct therapy alongside doxorubicin and paclitaxel was linked with a notable reduction in both paclitaxel-associated neuropathy and doxorubicin-induced cardiotoxicity. It was suggested that the ALA protective effect is attributed to ALA antioxidant activity through scavenging ROS, regenerating endogenous antioxidants including glutathione, vitamin E, and C and through its metal chelation activity. Furthermore, it was observed that the administration of ALA led to significant reductions in the levels of several key indicators, including malondialdehyde, which is a marker for oxidative stress resulting from lipid peroxidation and believed to be the primary mechanisms responsible for the development of neuropathy and chemotherapy-induced cardiotoxicity (Werida *et al.*, 2022). Another study assessing the protective effect of ALA against cisplatin induced toxicity reported that, ALA effectively reduced cisplatin-induced ROS production and increased cell viability in an inner ear cell line. It was observed that antioxidant activity of ALA was through the activation of the NRF2/HO-1 antioxidant pathway (Lee *et al.*, 2019). Furthermore, it was reported that cisplatin-induced hearing loss in the guinea pigs may be limited to some extent by the concomitant use of α -lipoic acid (Ozkul *et al.*, 2014). In addition, recent research reported that pre- and post-treatments with ALA in vitro and in vivo to cisplatin-treated mouse cochlea and HEI-OC1 culture cells showed that ALA contributes to protecting mitochondrial function by preventing ROS accumulation and inhibiting apoptotic cell death. More importantly post-treatment with ALA has showed restorative effect to cisplatin-induced ototoxicity (Kim *et al.*, 2018). Future studies should investigate the molecular mechanisms underlying the biphasic dose response of DOX mentioned earlier, particularly in relation to ROS production and DNA damage. Further research is also needed to explore combination therapies where DOX is paired with antioxidants, to reduce side effects while potentiating therapeutic effects in cancer treatment.

3.4 Cytotoxicity and ROS Measurements in Normal Human Kidney Cells (RPTEC)

In a continuation to the previous section normal human kidney cell line model, RPTEC, was used to assess toxicity level produced by the treatment of liposomal (DOX.AA.ALA), Doxosome, free (DOX.ALA.AA) and free DOX of same concentrations mentioned above (2.3, 4.7, 9.4, 18.7, 37.5 and 75 μ g/mL). MTS results revealed that liposomal (DOX.AA.ALA) had the highest cell viability with almost no significance changes for all concentrations used when compared to the untreated control. On the other hand, Doxosome, free (DOX.ALA.AA) and free DOX showed a marked decrease in cell viability with high significance level ($p \leq 0.0001$) specially in the low concentrations (2.3, 4.7 and 9.4 μ g/mL) when compared to the control group. It was also observed that low concentrations (2.3, 4.7 and 9.4 μ g/mL) of Doxosome and free DOX had the lowest level of cell viability indicating that those concentrations had a higher toxicity level to normal kidney cells (Figure 5C).

Additionally, ROS measurements were consistent with our earlier findings, where liposomal (DOX.AA.ALA) significantly showed the least % of ROS production in RPTEC which was followed by Dodosome, free (DOX.ALA.AA) and finally free DOX that showed a marked increase in ROS production, indicating it's potential to cause high level of nephrotoxicity (Figure 5D).

In summary, our cytotoxicity and ROS assessments have suggested that the presence of AA and ALA in free form and especially in liposomal form ameliorated the toxic effect of DOX on RPTEC cells through the antioxidant effect of AA and ALA, which has led to a marked decrease in ROS production

3.5 Cell Apoptosis Assay

Apoptosis assays were conducted to study whether the cytotoxic effects of liposomal (DOX.AA.ALA) were due to the induction of apoptosis or necrosis. Thus, to evaluate the incidence of apoptosis in the cancer cell lines, cells treated with liposomal (DOX.AA.ALA), liposomal (DOX), free (DOX.AA.ALA) and free DOX were assayed with Annexin V/7AAD double staining using flow cytometry. All formulations used the same concentration of DOX (2.3 µg/mL) which was selected based on the cytotoxicity assay as the lowest concentration showing the highest cell growth inhibition. The flow cytometry results showed that all formulations induced significant levels of late apoptosis in MCF-7 cells. It was observed that liposomal (DOX.AA.ALA) had the highest % of late apoptosis when compared to all other MCF-7 treated formulations (Figure 6). It was noteworthy to mention that liposomal (DOX.AA.ALA) significantly induced more apoptosis when compared to free (DOX.AA.ALA) and liposomal (DOX). This has indicated that liposomal drug carrier coloaded with antioxidants; AA and ALA further enhanced the apoptosis effect of DOX. Where both antioxidants had an anti-cancer effect, they both reduce hydrolysis and oxidation by donating two electrons that can neutralise the superoxide which causes lipid peroxidation of liposomes, regenerate DOX to its active form and finally ALA can regenerate AA to its active form where it can donate two electrons again (Figure 7). It was previously, reported that DOX nanoparticles coloaded with the antioxidant curcumin induced more apoptosis in treated HepG2 cells than free DOX, free DOX with curcumin and DOX nanoparticles (Zhao *et al.*, 2014). This has indicated that antioxidants have an apoptotic effect and have the potential to synergise DOX anti-cancer effect. It was also noted that MCF-7 treated cells in this study showed significantly greater levels of late apoptosis than early apoptosis, except for liposomal (DOX) that showed increased level of both early and late apoptosis. This could be explained through two suggested explanations; firstly, the MCF-7 cell line could be more liable and sensitive to the treatment than the other cell lines, secondly AA sensitised the cells towards treatment and increased the DOX uptake inside the cells leading to cell death. Also as mentioned previously, MCF-7 has exhibited differential susceptibility and response to anti-cancer drugs in comparison to MDA-MB-231 cell line, leading to differences in cell sensitivity to the inhibition rate. Specifically, MCF-7 cells were more responsive to DOX treatment at low drug concentrations than MDA-MB-231 cells, which could be the suggested explanation why MCF-7 cell line underwent late apoptosis with less early apoptosis percentage while MDA-MB-231 cell line had higher early apoptosis percentage when compared to MCF-7 cell line (Ghanbari *et al.*, 2022). Moreover, early apoptotic cells

can progress to late apoptotic cells if the early apoptosis persists due to an overload of dying cells and/or an impairment in phagocytosis (Poon *et al.*, 2010).

Insert Figures 6 and 7

On the other hand, a significant level of early and late apoptosis was detected for all MDA-MB 231 treated cells. Liposomal (DOX.AA.ALA) showed the highest level of early apoptosis while free (DOX.AA.ALA) showed the highest level of late apoptosis (Figure 6). It was also observed that the total (early and late) apoptosis of MDA-MB 231 cells induced by free (DOX.AA.ALA) and free (DOX) were higher than the total apoptosis induced by liposomal (DOX.AA.ALA). The results indicated that both free (DOX.AA.ALA) and free (DOX) induced more cell death. The liposomal (DOX.AA.ALA) didn't show the highest level of apoptosis index including early and late apoptosis when compared to free (DOX.AA.ALA) and free (DOX). However, the results are still acceptable and supporting our hypothesis since our novel liposomal (DOX.AA.ALA) showed the least toxic effect on normal human kidney cells in cytotoxicity assay. It was also observed that liposomal (DOX.AA.ALA) treated cells had a minimal level of necrosis % suggesting that liposomal (DOX.AA.ALA) induced cell death through apoptosis.

4 Conclusions

This study demonstrated the efficiency of a microfluidic system for the single-step fabrication of liposomes, achieving optimal drug encapsulation, stability, and size control without post-processing. The incorporation of ascorbic acid significantly enhanced stability and shelf life by reducing lipid oxidation, establishing its role in improving liposomal formulations. Biological assays confirmed that our novel liposomal DOX formulation exhibited superior cancer cell cytotoxicity and minimal nephrotoxicity compared to conventional and PEGylated formulations, suggesting its potential as a safer, targeted breast cancer therapy. Future studies are needed to validate these findings in vivo and optimize the formulation for broader applications.

5 Limitations

However, the study's limitations include its focus on in vitro assays, which may not fully replicate in vivo conditions. Further investigations are required to evaluate pharmacokinetics, biodistribution, and therapeutic efficacy in animal models. Future research could also explore optimizing liposome composition for other drug molecules and cancers, as well as integrating advanced targeting mechanisms such as ligand-based targeting or stimuli-responsive release systems.

6 Future perspective

Although there are FDA approved liposomes with anticancer drugs for breast cancer, there is still unmet clinical need for example to reduce the side effects associated with those nanomedicines, hence combinational nanotherapy may eliminate some of the issues/challenges. The findings from this study have several important implications for patient treatment, particularly for those undergoing chemotherapy for breast cancer. The novel liposomal formulation presented here displayed improved cytotoxicity toward breast cancer cells compared to free DOX or commercial liposomal formulations, suggesting that patients could potentially be offered a more effective cancer treatment. However, we additionally demonstrate that our formulation may minimise the adverse effects typically associated with

chemotherapy such as nephrotoxicity, which would likely improve the patient's overall quality of life and reduce long-term organ damage.

Our liposomal formulation showed excellent stability and a controlled, sustained drug release profile. With such favourable pharmacokinetics, this could translate into less frequent dosing for patients, improving compliance and more effective tumour suppression. Controlled drug release may allow for lower doses of doxorubicin to be used as the drug will remain active over a longer period, which could pave the way for personalized chemotherapy treatments tailored to individual patients based on their specific drug response.

The inclusion of antioxidants in this formulation seems to play a role in protecting normal cells by minimising ROS production, which in addition to reducing organ damage might also reduce other chemotherapy-related side effects, such as fatigue and hair loss that are often caused by the high levels of ROS generation. Reducing side effects while maintaining or improving efficacy has the potential to greatly improve the quality of life during treatment without impacting patient survival rates.

While this study focuses on breast cancer, our liposomal formulation could be applied to other cancers treated with doxorubicin or similar chemotherapeutic agents, which could lead to broader application and improved treatment for a wider range of patients. If validated in clinical trials, this formulation could represent a significant advancement for cancer treatment, with the potential to revolutionize the field of oncology using nanomedicine.

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(DOX.AA.ALA), and free DOX significantly reduced cell viability ($p \leq 0.0001$), particularly at low concentrations (2.3, 4.7, and 9.4 $\mu\text{g/mL}$), indicating higher toxicity to normal kidney cells. (D) ROS production in RPTEC treated with different drug formulations. ROS were measured after 24 h incubation with liposomal (DOX.AA.ALA), Doxosome, free (DOX.ALA.AA) and free DOX of different concentrations (2.3, 4.7, 9.4, 18.7, 37.5 and 75 $\mu\text{g/mL}$). Liposomal (DOX.AA.ALA) showed the lowest % ($p \leq 0.0001$) ROS production in RPTEC, followed by Doxosome and free (DOX.ALA.AA), while free DOX exhibited the highest ROS levels, indicating a greater potential for nephrotoxicity. Significance level is presented as (ns; no significance), (*; $p \leq 0.05$), (**; $p \leq 0.01$), (***; $p \leq 0.001$), and (****; $p \leq 0.0001$), (n=3, mean $\pm$ SD).

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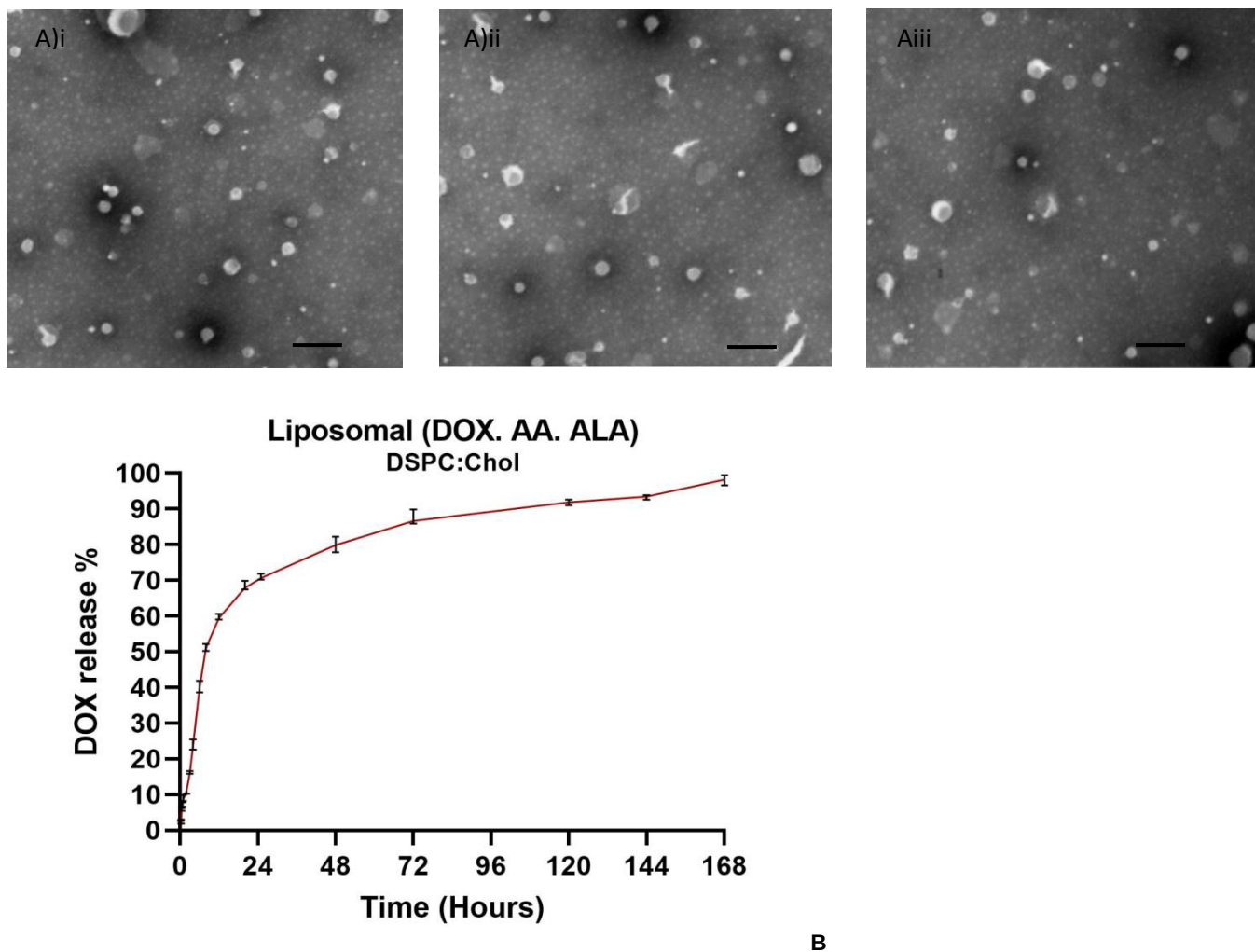


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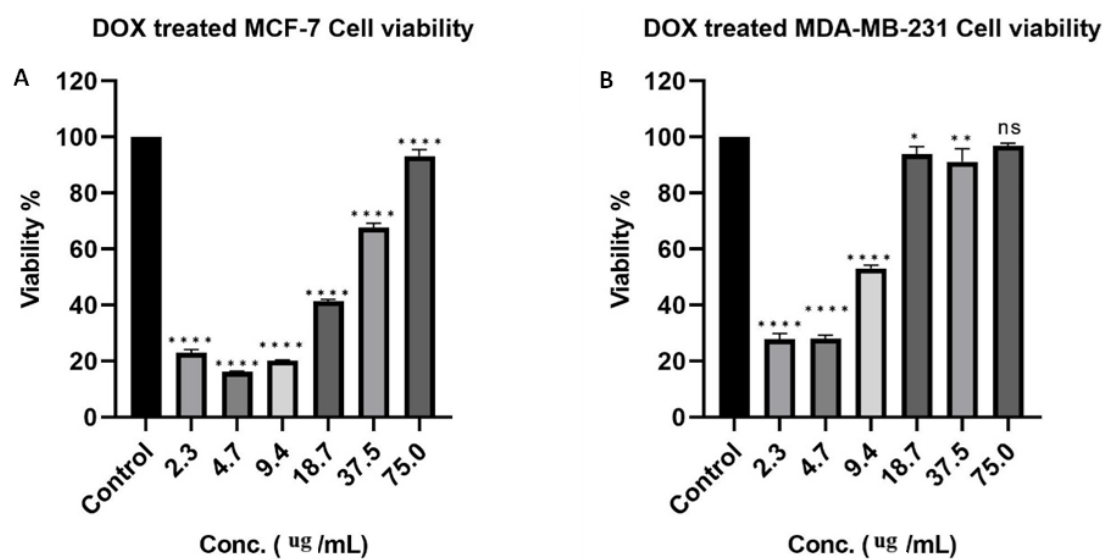


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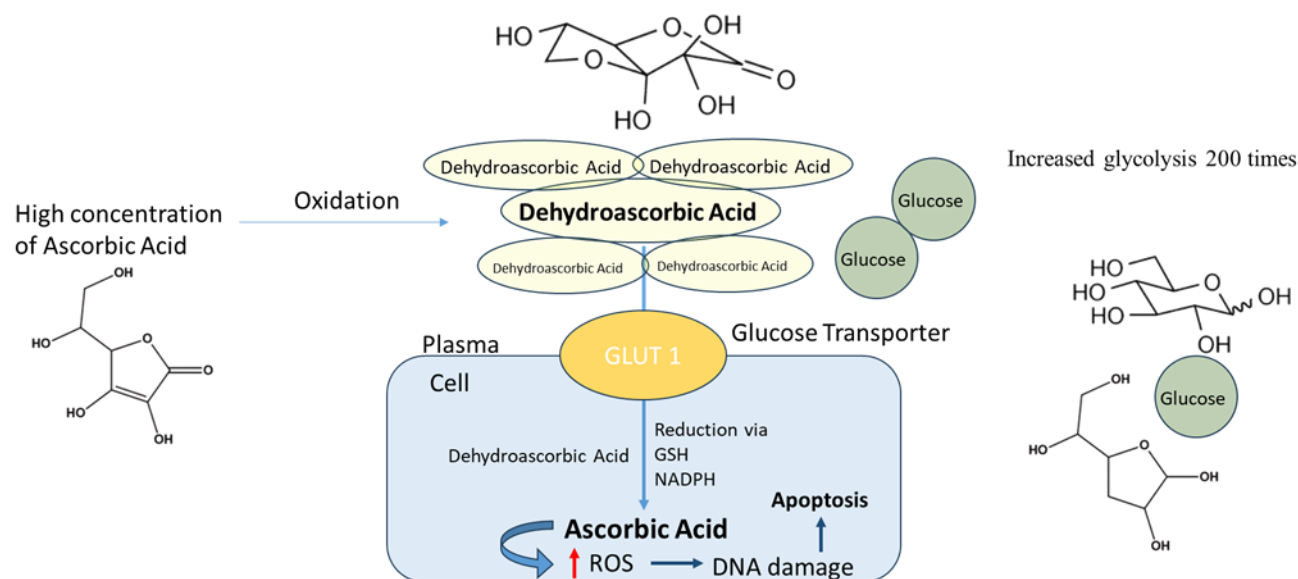
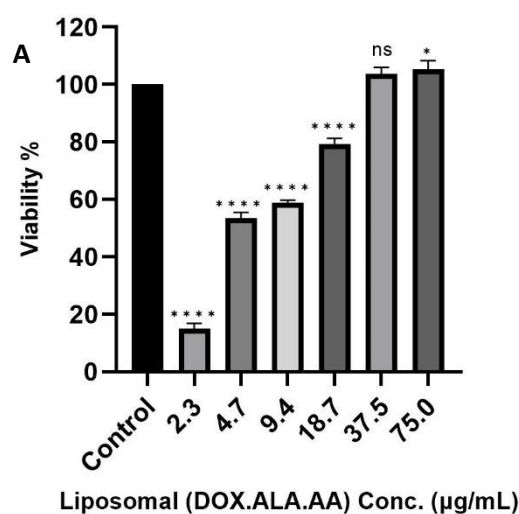
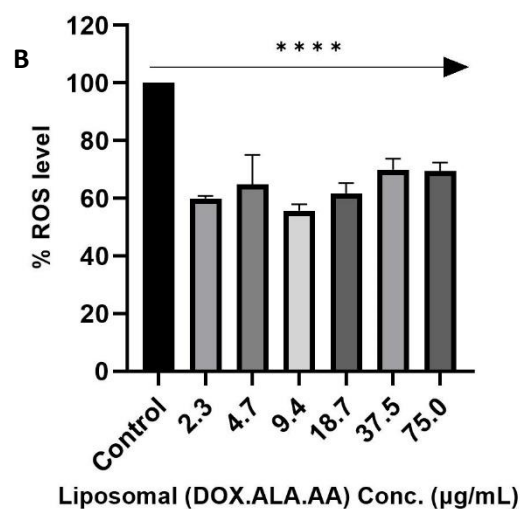


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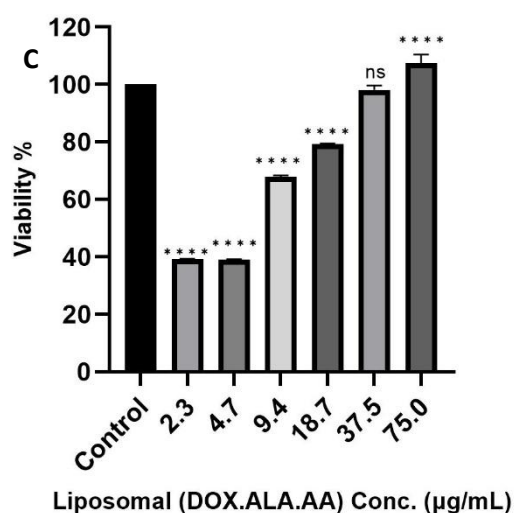
Liposomal (DOX.ALA.AA) treated MCF-7 Cell viability



ROS Production in liposomal (DOX.ALA.AA) treated MCF-7



Liposomal (DOX.ALA.AA) treated MDA-MB-231 cell viability



ROS Production in liposomal (DOX.ALA.AA) treated MDA-MB-231

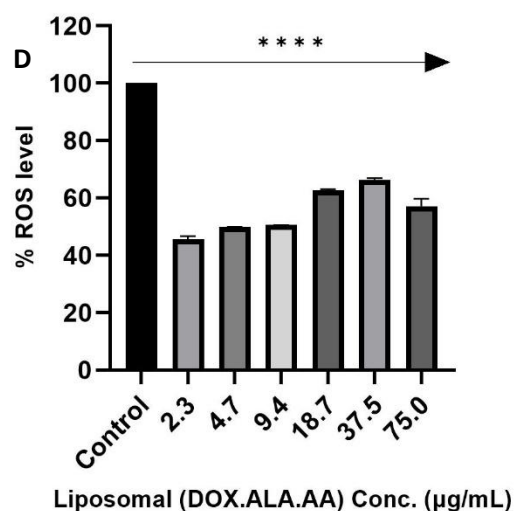


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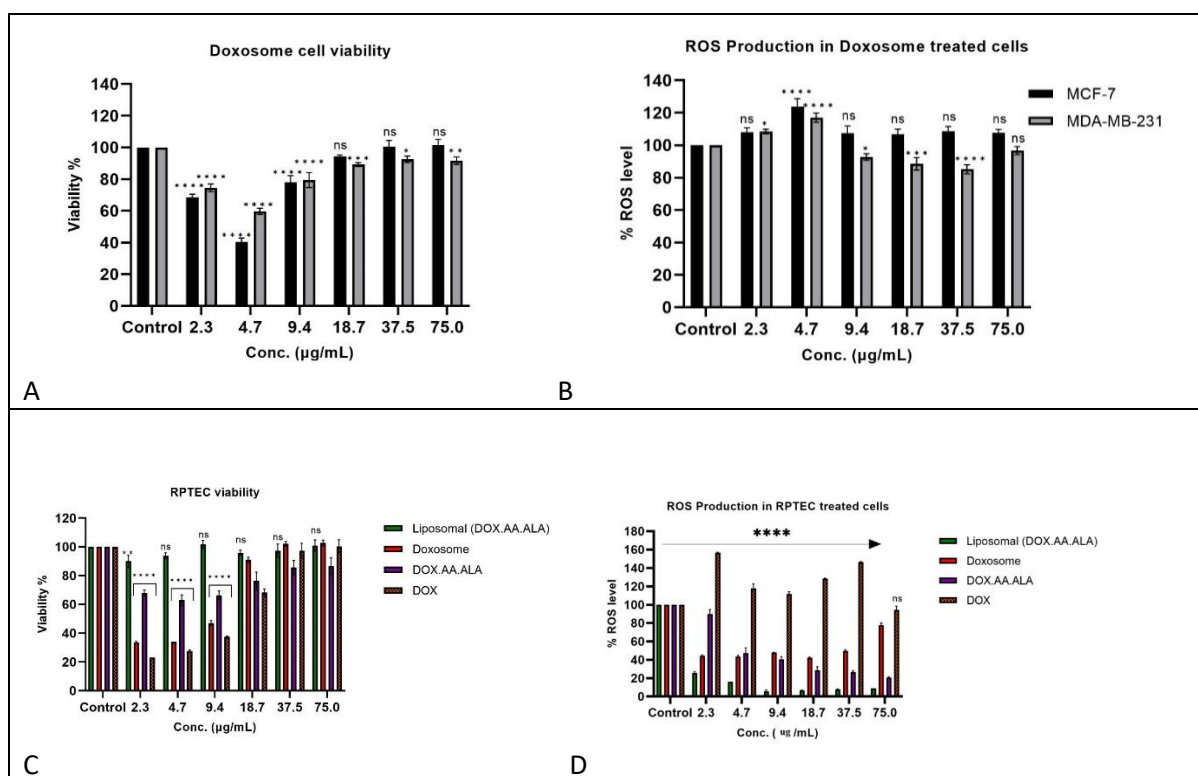


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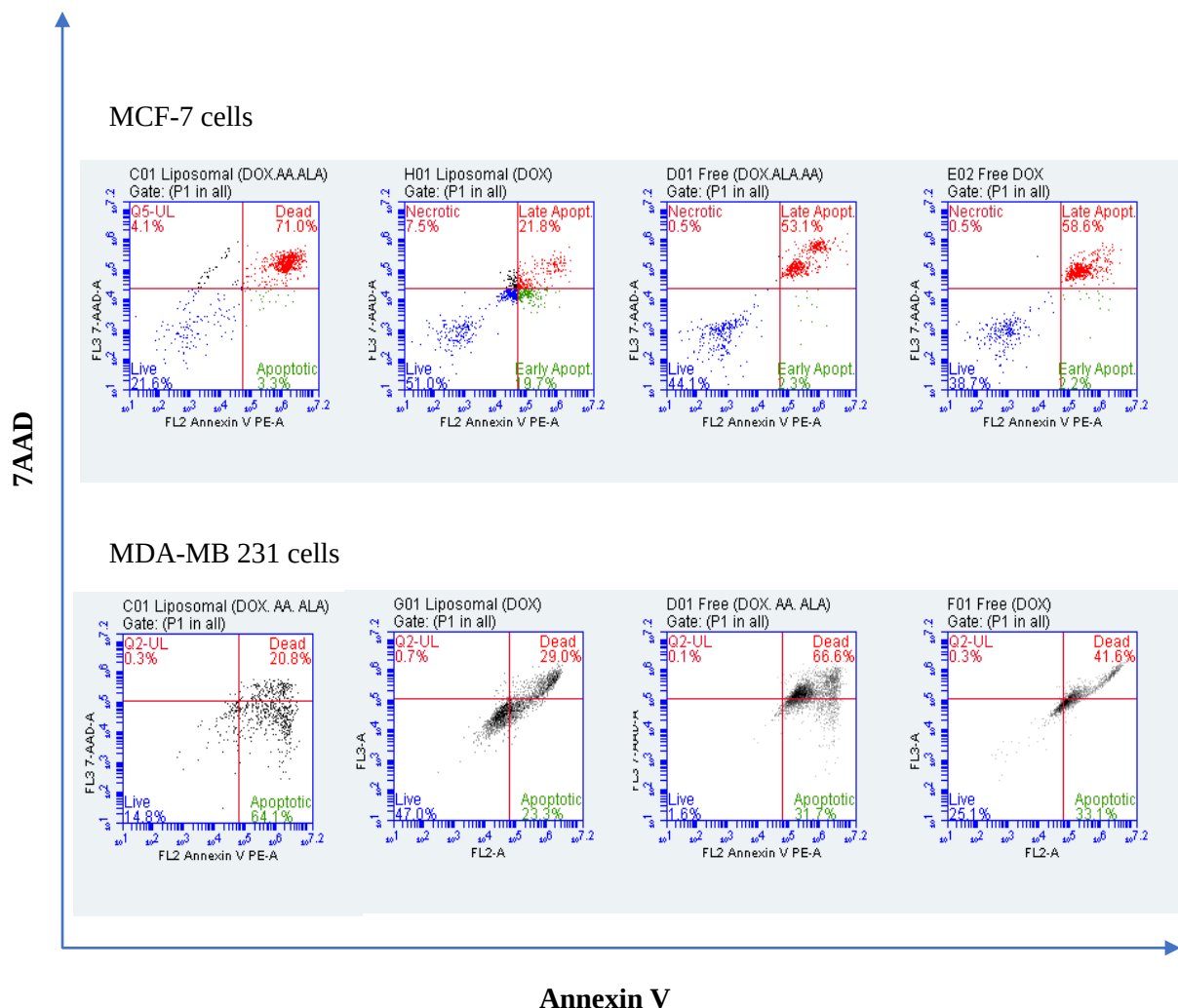


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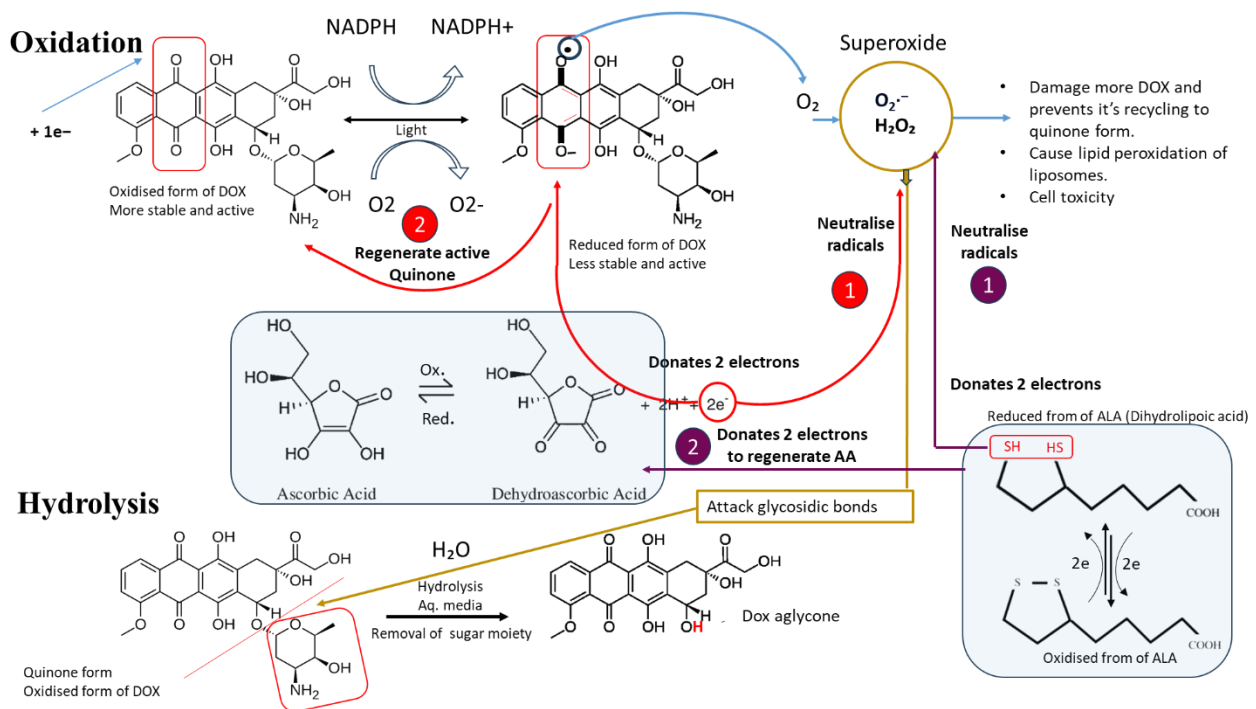


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Table 1. The experimental design for the preliminary study for producing the most suitable formulation. Different liposomal formulations have been prepared using different lipid: cholesterol concentration ratios and different microfluidic flow rate ratios between aqueous buffer, PBS: ethanol. The preliminary study showed that three formulations have been chosen from the initial prepared six formulations based on the mean diameter size and PDI. And based on the encapsulation efficiency using a mock drug, crystal violet. Lipid: cholesterol ratio (1:1) and PBS: ethanol flow rate ratio (5:1) have been chosen as the most ideal parameters to be used for further assessment in the study with DOX.

		Lipid: Cholesterol Ratio/ PBS: Ethanol Flow Rate Ratio								
Plain liposome (DSPC) ^a	1:1/ 1:1		Liposomal (AA, CV) ^b	1:1/ 5:1		Liposomal (AA) ^c	1:1/ 5:1		Liposomal (DOX, AA, ALA) ^d	1:1/ 5:1
	1:1/ 2:1			2:1/ 2:1				2:1/ 2:1		
	1:1/ 5:1			2:1/ 5:1				2:1/ 5:1		
	2:1/ 1:1									
	2:1/ 2:1									
	2:1/ 5:1									

^aLiposomes made of 1,2-distearoyl-sn-glycero-3-phosphocholine (2.5 mg/ml)

^bLiposomes (Ascorbic acid (50 mg/ml) and Crystal violet, CV (0.05 mg/ml))

^cLiposomes (Ascorbic acid (50 mg/ml))

^dLiposomes (Doxorubicin (0.05 mg/ml), Ascorbic acid (50 mg/ml), and Alpha lipoic acid (50 mg/ml))

Table 2. A summary for all physicochemical characterisation obtained for all prepared formulations. A comparison between the data obtained from preparing different formulations using different ratios (DSPC: cholesterol/ PBS buffer: ethanol microfluidics flow rate ratio). The preliminary data suggested that the formulation (DSPC: cholesterol/ PBS buffer: ethanol microfluidics flow rate ratio) of (1:1/ 5:1) had the highest encapsulation efficiency for both the mock drug (CV) and DOX. Hence, the selection of (1:1/ 5:1) ratios to be used for further assessment and application in the study. All values represent mean $\pm$ standard deviation (SD) for three independent replicates (n=3).

Lipid:cholesterol/ PBS: ethanol microfluidics flow rate ratio															
1:1/ 5:1					2:1/ 2:1					2:1/ 5:1					
Liposomes	Mean diamet er (nm)	PDI	ZP (mV)	EE (%)	DOX Load (w/w)	Mean diamet er (nm)	PDI	ZP (mV)	EE (%)	DOX Load (w/w)	Mean diamet er (nm)	PDI	ZP (mV)	EE (%)	DOX Load (w/w)
Plain liposomes	71.6 $\pm$ 5.0	0.2 $\pm$ 0.0 3	-0.4 $\pm$ 0.0 3	-		68.5 $\pm$ 3.2	0.1 $\pm$ 0.03	-0.5 $\pm$ 0.1	-		62.0 $\pm$ 4.5	0.2 $\pm$ 0.1	-0.23 $\pm$ 0.3	-	
Liposomal (AA.CV) ^a	75.5 $\pm$ 0.1	0.1 $\pm$ 0.0 2	-0.6 $\pm$ 0.3	76. 0 $\pm$ 2.1		73.5 $\pm$ 0.5	0.05 $\pm$ 0.01	1.1 $\pm$ 0.4	22. 0 $\pm$ 1. 5		51.2 $\pm$ 0.2	0.10 $\pm$ 0.01	-5.6 $\pm$ 1.1	35.0 $\pm$ 0.7	
Liposomal (AA) ^b	66.6 $\pm$ 4.0	0.2 $\pm$ 0.0 3	-0.3 $\pm$ 0.6	-		71.9 $\pm$ 1.5	0.06 $\pm$ 0.02	0.2 $\pm$ 0.4	-		58.2 $\pm$ 5.5	0.23 $\pm$ 0.05	-3.4 $\pm$ 1.7	-	
Liposomal (DOX.AA.ALA) ^c	79.0 $\pm$ 0.3	0.1 $\pm$ 0.0 1	-4.0 $\pm$ 1.2	97. 0 $\pm$ 0. 6	0.5mgDOX/105. 5mg total content	171.0 $\pm$ 1.3	0.05 $\pm$ 0.03	-0.4 $\pm$ 0.5	13. 0 $\pm$ 1. 0	0.5mgDOX/10 8mg total content	101 $\pm$ 0.6	0.05 $\pm$ 0.02	-2.4 $\pm$ 0.7	52.0 $\pm$ 3.0	0.5mgDOX/10 8mg total content

^aLiposomal (Ascorbic acid and Crystal violet)

^bLiposomal (Ascorbic acid)

^cLiposomal (Doxorubicin, Ascorbic acid, and Alpha lipoic acid)

Table 3. Storage stability study for 5 months (M1 to M5) at 4°C of plain liposomes made of DSPC/Cholesterol (1:1) and with 5:1 microfluidic flow rate ratio, FRR, without the incorporation of antioxidants and drugs. The table presents the vesicles mean diameter and polydispersity index (PDI), the data suggest that the storage led to significant increase ($p < 0.05$) in the mean diameter of liposomal vesicles which could be explained by formulation instability caused by hydrolytic degradation and oxidative degradations (Boggula *et al.*, 2022). Data represent mean $\pm$ standard deviation ($n = 3$).

Storage Time in Months (M)	Mean Diameter (nm)	PDI
Liposomes direct from the system	71.6 $\pm$ 5.0	0.16 $\pm$ 0.03
M1	117.5 $\pm$ 2.8	0.26 $\pm$ 0.01
M2	147.5 $\pm$ 3.8	0.44 $\pm$ 0.02
M3	177.3 $\pm$ 10.1	0.43 $\pm$ 0.04
M4	209.6 $\pm$ 13.2	0.57 $\pm$ 0.09
M5	519.0 $\pm$ 197.0	0.56 $\pm$ 0.15

Table 4. Storage stability study for 5 months (M1 to M5) at 4°C of liposomal ascorbic acid (AA) made of DSPC/Cholesterol (1:1) and with 5:1 microfluidic flow rate ratio, FRR. The table presents the mean diameter and polydispersity index (PDI) for liposomes co-loaded with AA only. The results proposed that encapsulation of AA stabilised the liposomal formulation, maintained the vesicle size compared to plain liposomes, AA reduced oxidation, fusion, and aggregation during storage. Data represent mean $\pm$ standard deviation ($n = 3$).

Storage Time in Months (M)	Mean Diameter (nm)	PDI
Liposomes direct from the system	66.6 $\pm$ 4.0	0.18 $\pm$ 0.03
M1	79.8 $\pm$ 0.5	0.11 $\pm$ 0.01
M2	78.4 $\pm$ 1.2	0.10 $\pm$ 0.02
M3	80.7 $\pm$ 1.5	0.21 $\pm$ 0.01
M4	78.4 $\pm$ 5.7	0.31 $\pm$ 0.10
M5	98.0 $\pm$ 2.6	0.41 $\pm$ 0.03

Table 5. The storage stability study for 11 months (M1 to M11) at 4°C of liposomal doxorubicin, ascorbic acid and alpha lipoic acid (DOX.AA.ALA) made of DSPC/Cholesterol (1:1) and with 5:1 microfluidic flow rate ratio, FRR, the table presents the vesicles mean diameter and polydispersity index (PDI). One way ANOVA test showed no statistical difference at ($p > 0.05$) between the mean diameter of fresh liposomes obtained directly from the system and the mean diameter of liposomes after storage for 6 months, M1 to M6. Then, there was a statistical difference ($p < 0.05$) after 6 months storage, M7 to M11. However, vesicles mean diameter till M11 did not exceed 150 nm which is still an acceptable size range for good internalization and retention in the tumour interstitium (Olusanya et al., 2018). Regarding the PDI, its noteworthy to mention that all PDI results (M1 to M11) were below 0.3, indicating that liposomes were highly monodispersed (Refai et al., 2017; Danaei et al., 2018). This liposomal (DOX.AA.ALA) preparation showed the highest DOX encapsulation efficiency, EE, with a slightly negative zeta potential, indicating its stability. Data represent mean $\pm$ standard deviation (n = 3).

Storage Time in Months (M)	Mean Diameter (nm)	PDI	ZP (mV)	EE (%)	Drug load (w/w)
Liposomes direct from the system	79.0 $\pm$ 0.3	0.13 $\pm$ 0.01	-4.0 $\pm$ 1.2	97.0 $\pm$ 0.6	0.5mg DOX/105.5mg total content
M1	76.1 $\pm$ 0.8	0.10 $\pm$ 0.01	-3.9 $\pm$ 1.1	-	
M2	72.1 $\pm$ 0.2	0.12 $\pm$ 0.01	-5.3 $\pm$ 0.6	-	
M3	72.7 $\pm$ 0.7	0.13 $\pm$ 0.01	-2.5 $\pm$ 0.7	-	
M4	73.2 $\pm$ 0.5	0.12 $\pm$ 0.01	-4.6 $\pm$ 1.1	-	
M5	71.7 $\pm$ 0.3	0.12 $\pm$ 0.01	-6.1 $\pm$ 2.3	-	
M6	81.9 $\pm$ 1.0	0.18 $\pm$ 0.01	-3.8 $\pm$ 0.5	-	
M7	95.9 $\pm$ 8.5	0.19 $\pm$ 0.07	-2.4 $\pm$ 1.6	-	
M8	95.2 $\pm$ 0.9	0.19 $\pm$ 0.02	-5.2 $\pm$ 0.9	-	
M9	90.6 $\pm$ 0.5	0.18 $\pm$ 0.01	-6.2 $\pm$ 2.0	-	
M10	105.3 $\pm$ 8.9	0.25 $\pm$ 0.03	-6.0 $\pm$ 2.8	-	
M11	112.1 $\pm$ 15.6	0.09 $\pm$ 0.05	-7.1 $\pm$ 1.5	-	

