Doxorubicin-loaded multivesicular liposomes (Depofoams) as a sustained release carrier intended for locoregional delivery in cancer treatment: Development, characterization, and cytotoxicity evaluation

Supplementary File

1. Materials and method

1.1 Materials

DOX, chloroform, methanol, orthophosphoric acid and acetonitrile (HPLC-grade) were obtained from Merck (Germany, Darmstadt). Purified egg phosphatidylcholine (EPC), dipalmitoyl phosphocholine (DPPC) and distearoyl phosphocholine (DSPC) were supplied by Lipoid GmbH (Switzerland). Chol (purity >99%), L-lysine free-base and triolein (TO), dicetyl phosphate (DCP) and sucrose were purchased from Sigma–Aldrich (Germany). Cellulose dialysis bag (molecular weight cutoff 12,000 Da) was purchased from BioGene (USA). MCF-7, 4T-1 and normal human fibroblast cells were procured from Cell Bank of Pasteur Institute (Iran). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin (pen/strep), and trypsin-ethylenediaminetetraacetic acid (EDTA) all supplied from Gibco BRL (USA). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) provided by Sigma–Aldrich (Germany).

1.2 Experimental Design

Independent variables, levels of factorial design and their range are presented in Appendix 1.

1.3 *In vitro* release studies

A sample of each formulations was put into a dialysis bag (molecular weight cut-off of 12 kDa) and dialyzed against 50 ml of PBS (pH 7.4). The release studies were performed at 37 ℃ under 50 rpm magnet stirring and light protection (2). At predetermined time intervals, samples of 0.5 ml were taken and refilled with an equal volume of fresh PBS to maintain sink condition. Drug concentration was analyzed via a previously reported validated high-performance pressure liquid chromatography (HPLC) method (3). Briefly, The chromatographic separation was performed on

a C18 PerfectSil column (4.6 × 150 mm, 5 µm particle size, MZ-Analysentechnik, Mainz, Germany) using a mobile phase consisting of acetonitrile and water (32: 68, v/v), adjusted to pH 2.6 with orthophosphoric acid and a flow rate of 1 ml/min. The column temperature was maintained at 35 °C and excitation and emission wavelengths were set at 475 and 555 nm, respectively.

1.4 Cytotoxicity assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay evaluates viable cells to determine the cytotoxicity of drugs in various concentrations (4). The cytotoxicity of the optimized formulation (DepoDOX) was investigated on normal human fibroblast cells, human breast cancer cell line (MCF-7) and murine breast cancer cell line (4T-1) cells. In short, the cells were grown in RMPI 1640, supplemented by 10 % FBS and 2% pen/strep (penicillin and streptomycin) solution and incubated at 37 ℃ in a 5 % CO2 atmosphere. The cells were seeded onto 96-well plates at a density of 15,000 cells per well (Costar ®, USA) and incubated for 24 h. Then, the assay cells were exposed to free DOX (0.5–30 µg/ml), DepoDOX or the related empty MVLs for 24, 48 and 72 h. The cells treated with the medium were also used as control. After washing the cells with PBS and adding 100 µL of the culture medium, the MTT solution (20 µL of 5 mg/ml in PBS) was applied to each well, and the cells were incubated for another 2 h. The absorbance was recorded at 490 nm with an enzyme-linked immunosorbent assay (ELISA) plate reader (ELX800, Biotek, USA). The half maximal inhibitory concentration (IC50) values of free DOX and DepoDOX were calculated. In addition, cell viability for each group was determined using the following equation:

Cell viability = (Mean absorbance of test wells - Mean absorbance of control wells)/ (Mean absorbance of untreated wells - Mean absorbance of control wells) × 100

1.5 Hemolysis assay

healthy human blood samples were placed in test tubes containing sodium citrate (3.8% wt.) at a volume ratio of 9:1, diluted with normal saline (4:5, v:v), and centrifuged at 2500 rpm for 10 minutes. After removing the supernatant, erythrocytes were washed with normal saline three times until the supernatant was clear. The collected red blood cells (RBC) were diluted with normal saline (1:10, v: v), and 0.5 ml of the cell suspension was incubated with 2 ml of MVLs (DepoDOX

MVL formulation) or free DOX solution for 1 h at 37 °C. Following the incubation, the samples were centrifuged at 3000 rpm for 5 minutes, and the supernatant was analyzed using UV-vis spectrophotometry at 545 nm. 0.5 ml of the RBC suspension diluted in distilled water and saline diluted blood were used as the positive and negative control, respectively. The following equation was used to calculate the hemolysis percentage.

Hemolysis index (%) = $(ABS_{sample} - ABS_{negative\ control}) / (ABSpositive\ control - ABSnegative\ control) \times 100$

2. Results

2.1 Preliminary studies and characteristics of the prepared MVLs

According to the percent drug release data (Table 2), all formulations prepared in the first stage were able to properly control the drug release rate. About 32- 43% of the loaded drug was released over the first 6 h, after then, the release of DOX became slow as the highest drug release percentage (DR%) over 120 h was 70% . Through an overall evaluation of the DR% over the first 6 h, it can be found that MVLs with a higher Chol/PL ratio (DSPC 2.5, DPPC 2.5, EPC 2.5) have faster initial drug release compared to similar formulations with a lower ratio (DSPC 1.5, DPPC 1.5, EPC 1.5). The latter formulations displayed relatively similar initial (0-6 h) release profiles, however, at later times, DSPC1.5 and DPPC 1.5 showed much slower release rates than EPC 1.5. As shown in Table 2, DR% in the duration of 72-120 h for DSPC 1.5, DPPC 1.5 and EPC 1.5 were 2.68%, 2.76%, and 9.87%, respectively. Based on the release results, EPC was chosen for further optimization of DOX containing MVLs

2.2 Experimental design and data analysis

Analysis of variance (ANOVA) was applied to determine and understand the significance of the effects of each variable and their interactions. The correlation of the effect of the variables on the responses was expressed by the polynomial equation. Model summary statistics are summarized in Appendix 2. The high values for the adjusted and predicted R-squared, as well as insignificant lack of fit for all responses ($p > 0.05$), indicate that the model fits the data well. The effect of variables on each of three responses and a comparison of the impact of all factors at a particular point in the design space were further analyzed by applying the pareto diagram (Appendix 5) and the perturbation diagram (Appendix 6), respectively.

2.3 Morphology, zeta potential and FTIR spectroscopy

MVLs morphology at ×400 and ×1000 magnification with an optical microscope were spherical, honeycomb-like structure of tiny chambers (Appendix 7).

The characteristic peaks of DOX as H-N stretching vibrations related to the amine structure (1615 and 3450 cm^{-1}), the H-O tensile vibrations (3330 cm^{-1}) and C=O stretching vibrations (1745 cm^{-1}) are present in the spectrum of pure DOX and are consistent with previous reports (5-8). EPC showed characteristic peaks corresponding to stretching vibrations of P–O–C and P=O (1080 cm^{-1} and 1232 cm^{-1}, respectively), C=O stretching, (1736 cm^{-1}), and quaternary nitrogen (3,419 cm^{-1}) (9). The peaks in the Chol spectrum that are located between 2940 and 3380 cm^{-1} are related to C─H stretching vibrations of methyl groups and cyclic hydrocarbons vibrations (10). Triolein was found to have four strong bands at 2923, 2854, 1743, and 1164 cm^{-1}, which were related to the asymmetric and symmetric CH2 stretching, C=O and C-O-C stretching in triglyceride molecules, respectively (11). Bands between 3000 and 2800 cm^{1} and between 1700-1600 cm^{-1} correspond to the CH2 and CH3 groups and O=P-H group of DCP, respectively (12). The peaks appeared in the spectrum of DepoDOX were almost identical to the empty MVLs, indicating the absence of any interaction between the drug and MVL ingredients. Drug peaks were not observed in the DepoDOX spectrum, which may be probably due to the low drug to lipid ratio in this formulation (Appendix 8).

4.6. Stability of DepoDOX under the storage conditions

The stability of DepoDOX in terms of the particle size, span value, zeta potential and EE% was evaluated at 4 °C for 4 weeks and the results are shown in Appendix 3. As shown, the formulation was stable within 4 weeks and no significant difference was observed in the mentioned characteristics.

4.7. Evaluation of cytotoxicity using the MTT method

The cytotoxic activity of the selected formulation in comparison with the free drug was evaluated against 4T1, MCF7 and fibroblast cell lines, and the IC50 results are shown in Appendix 4.

Appendix 1: Independent variables and respective levels of factorial design for preparation of MVLs

Independent Variables	Unit	Range	Design levels	
			-1	**+1**
Chol/EPC (A)	-	1-2	1	2
TO (B)	%	15-25	15	25
L/D (C)	-	10-30	10	30

Abbreviations: Chol/EPC: cholesterol to egg phosphatidylcholine; L/D: lipid to drug molar ratio; TO: triolein.

Appendix 2: Statistical results obtained from experimental design

	EE%	DR_{6h}%	DR_{72h}%
Model	Significant (0.0062)	Significant (0.0021)	Significant (0.0027)
Curvature	Significant (0.0187)	Significant (0.0075)	Significant (0.0026)
Lack of Fit	Not Significant (0.0587)	Not Significant (0.7863)	Not Significant (0.9339)
R^2	0.9315	0.9809	0.9784
Adjusted R^2	0.8629	0.9524	0.9459

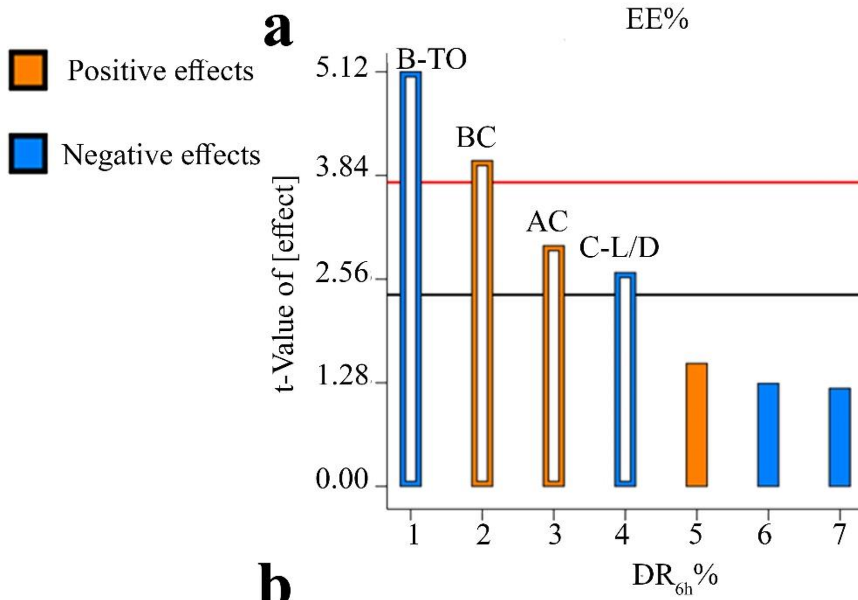
Appendix 3: Stability of the DepoDOX stored at 4 °C (mean ± SD, n = 3).

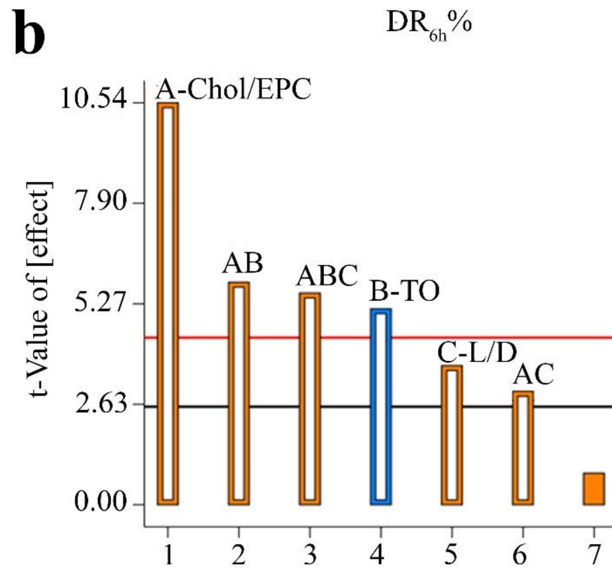
Parameters	Time (week)			
	0	**1**	**2**	**4**
Size (µm)	9.72 ± 0.23	9.33 ± 0.17	8.95 ± 0.37	9.11 ± 0.26
Span value	1.87 ± 0.01	2.12 ± 0.13	1.69 ± 0.11	2.08 ± 0.07
Zeta potential (mV)	-36.3 ± 0.01	nd[a]	nd	-33.7 ± 0.07
EE (%)	83.9 ± 0.6	83.1 ± 0.30	82.9 ± 0.30	82.5 ± 0.40

[a] nd: was not determined

Appendix 4: IC50 values of free DOX and DepoDOX on 4T1, MCF-7 and fibroblast cells (mean ± SD, n = 3).

Cell line	IC50 (µg/ml)	
	DOX	DepoDOX
4T-1	1.91 ± 0.34	22.25 ± 0.23
MCF-7	5.17 ± 0.12	23.21 ± 0.13
Fibroblast	14.89 ± 1.03	16.54 ± 1.67


a
EE%
Positive effects
Negative effects
B-TO
BC
AC
C-L/D
5.12
3.84
2.56
1.28
0.00
t-Value of [effect]
1
2
3
4
5
6
7


b
DR6h%
A-Chol/EPC
AB
ABC
B-TO
C-L/D
AC
10.54
7.90
5.27
2.63
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t-Value of [effect]
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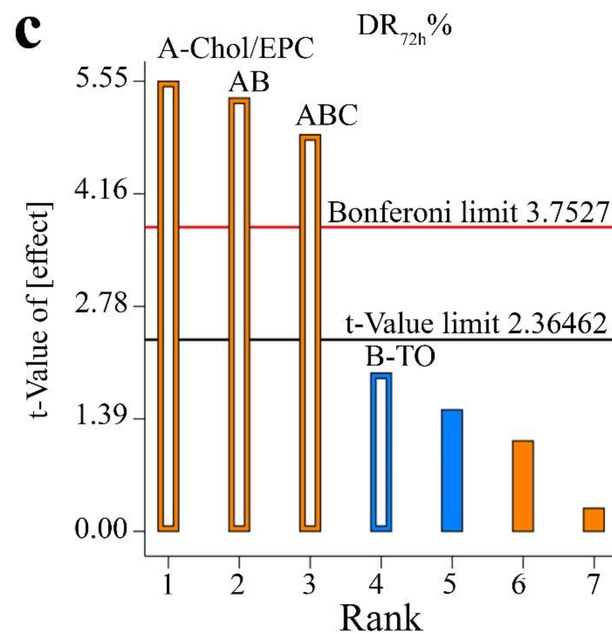
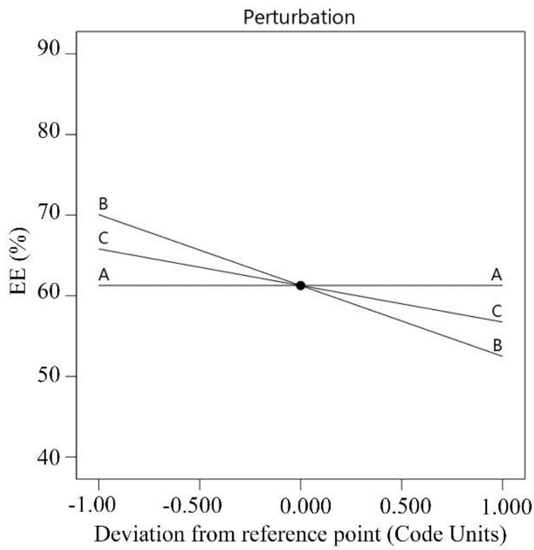
2
3
4
5
6
7

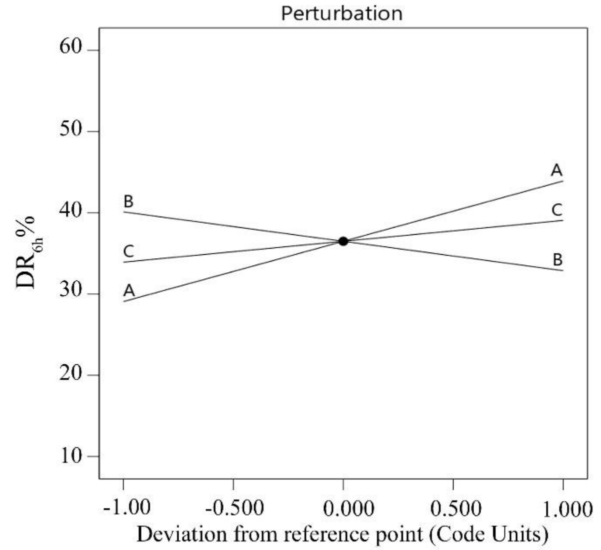

c
DR72h%
A-Chol/EPC
AB
ABC
Bonferoni limit 3.7527
t-Value limit 2.36462
B-TO
5.55
4.16
2.78
1.39
0.00
t-Value of [effect]
1
2
3
4
5
6
7
Rank

Appendix 5: Pareto charts of the analyzed effects for (A) EE (%), (B) DR_{6h} % and (C) DR_{72h} % of the DOX loaded MVLs recommended by experimental design

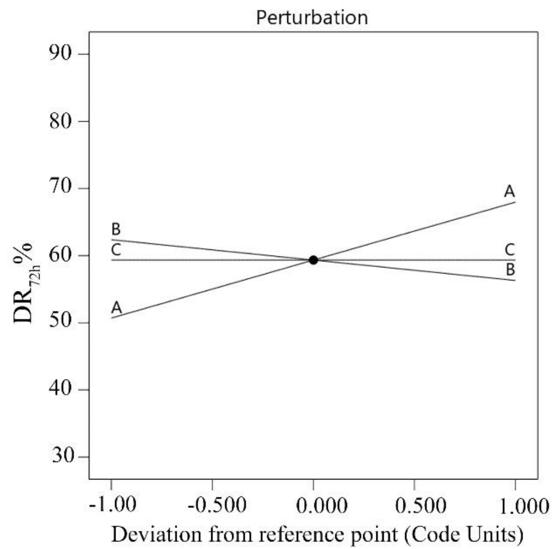
a



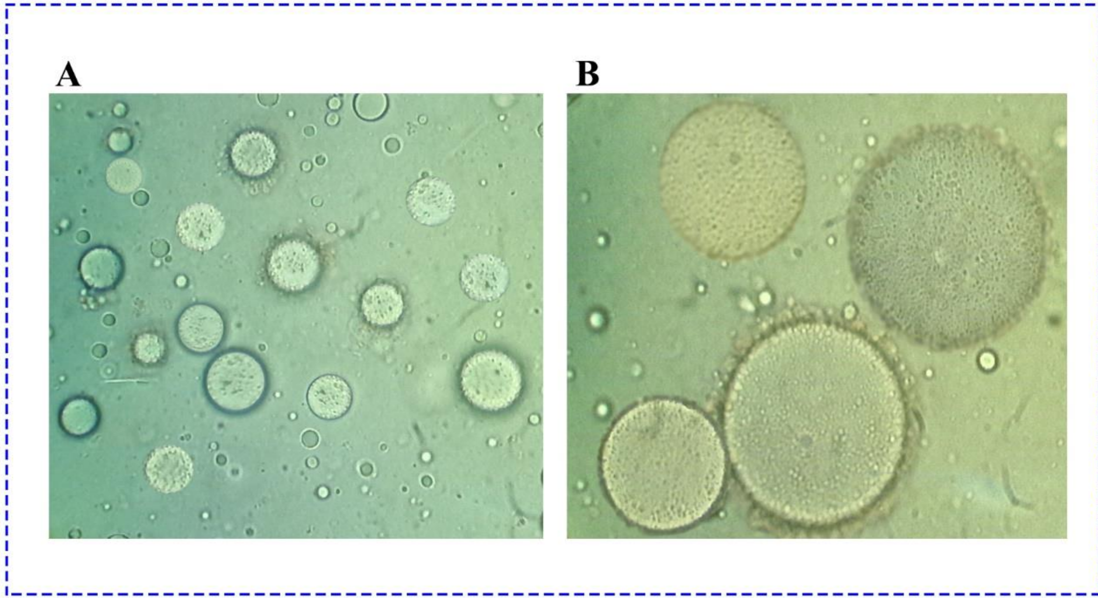
b



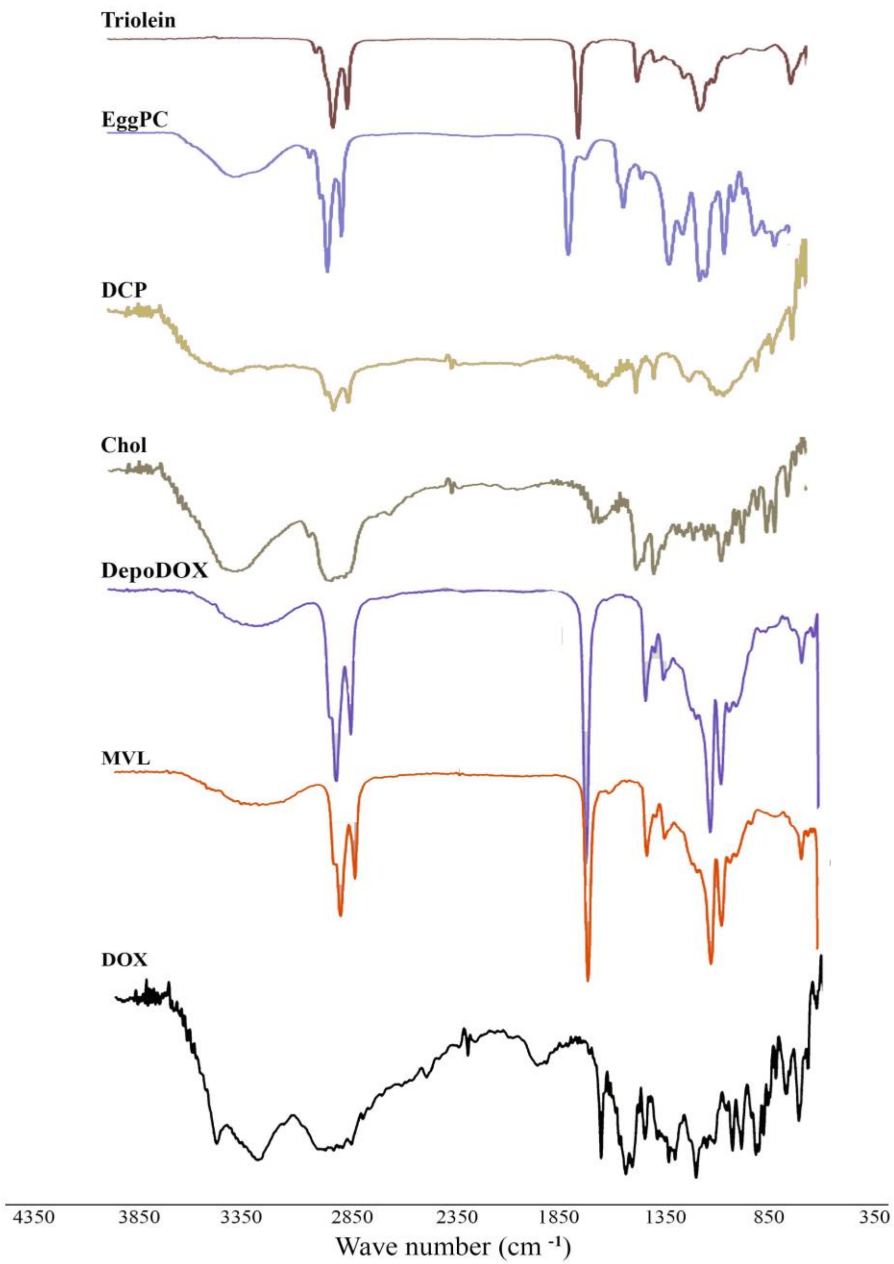
c



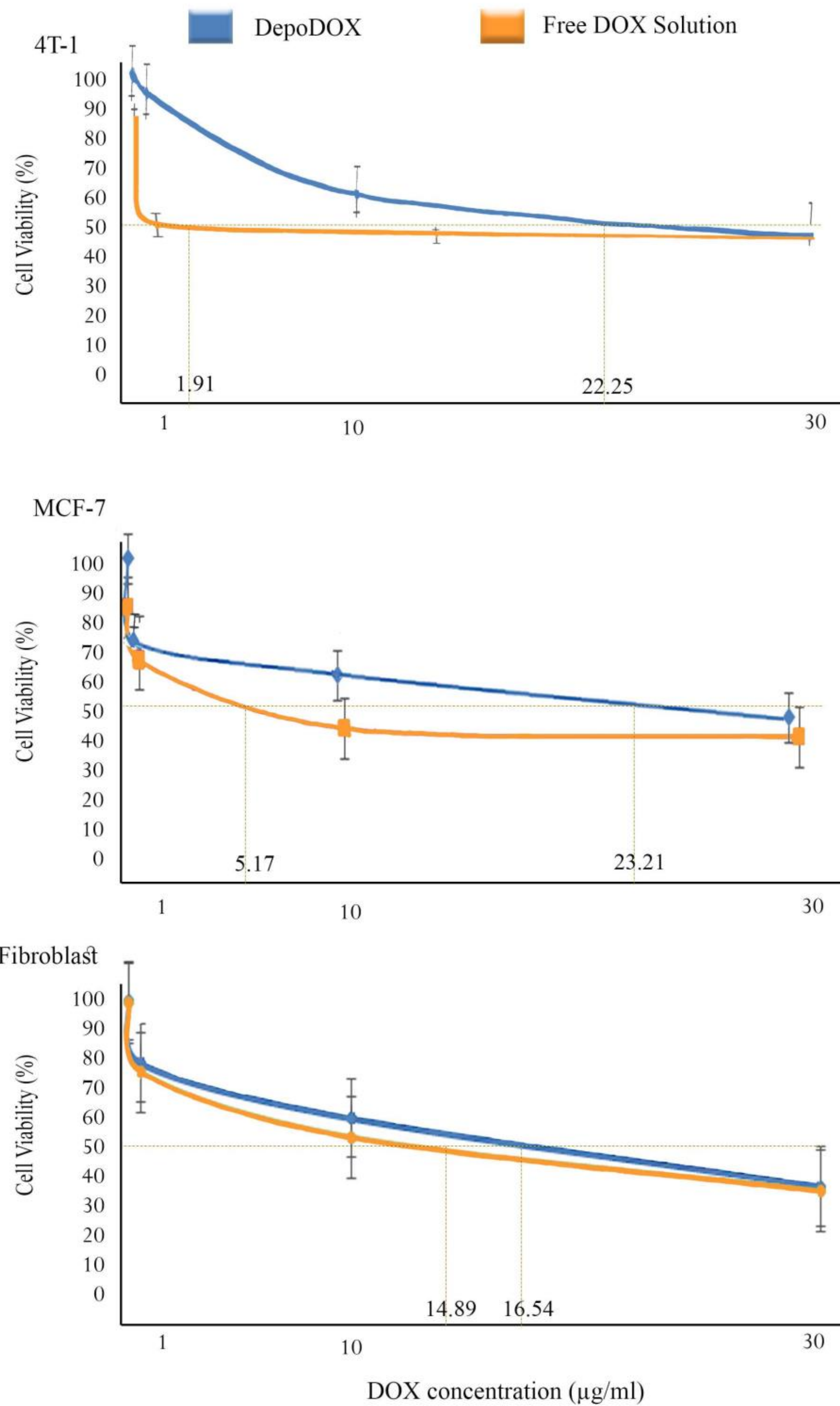
Appendix 6: Perturbation plots of different responses: (A) Chol to EPC molar ratio, (B) TO%, and (C) lipid to drug molar ratio



Appendix 7: Microscopic pictures of MVLs taken by a light microscope. (A) ×400 magnification B) ×1000 magnification



Appendix 8: FTIR spectra of EPC, Chol, TO, DCP, free DOX, empty MVL and DepoDOX formulation.



Appendix 9: Cell viability of 4T1, MCF7 and human fibroblast cells after incubation with free DOX solution and DepoDOX at concentrations ranging from 0.5 to 30 µg/ml

References

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