Pluronic F127/Doxorubicin microemulsions: Preparation, characterization, and toxicity evaluations

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Abstract

The development of drug delivery systems minimizing the side effects of conventional chemotherapy is one of the major challenges in the field of biomaterials for cancer treatment This work reports the formulation and characterization of oil-in-water F127 microemulsions to enhance the bioavailability of doxorubicin (DOX). The Density Functional Theory (DFT) calculations at the M06-2X level of theory were done to study the interaction details of DOX with ethyl butyrate, sodium caprylate, and one unit of the polymeric chain of surfactant F127 in water solution, which are used in the synthesis process. Specifically, the quantum theory of atoms in molecules (QTAIM) analysis was performed to determine the nature of interactions. The highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) energies were calculated to show the direction of charge transfer within each complex. Furthermore, the Natural Bond Orbital (NBO) analysis was performed on the studied systems.

The size of DOX microemulsion was about 7.0 nm by dynamic light scattering analysis. *In vitro* toxicity of standard DOX and DOX-loaded microemulsions were assessed on MCF-7 and C152 (malignant) and HUVEC (non-malignant) cell lines. Intracellular lactate dehydrogenase (LDH) leakage was evaluated as an indicator of membrane integrity and cell viability. *In vitro* assessments revealed that F127/DOX microemulsions caused substantial morphological changes and greater cytotoxic effects than standard DOX. Besides, F127/DOX microemulsions might trigger independent signaling pathways. F127/DOX microemulsions were injected intraperitoneally at 12 and 24 mg/kg into rats. The free (bulk) DOX group induced severe histopathological changes and significant increases in serum kidney markers and serum liver enzymes. The 24 mg/kg dose of F127/DOX microemulsions also induced fatty changes and elevation of serum liver enzymes and creatinine. Overall, this new drug delivery system formulation shows promise for cancer treatment and deserves to be further studied in the future.
KEYWORDS: Biomaterials, nanomaterials, polymers, drug release

1. Introduction

Cancer is among the prevalent causes of morbidity, accounting for one-quarter of all deaths globally [1]. Doxorubicin (DOX), also known as adriamycin, is a commonly used and highly potent chemotherapeutic drug for patients affected by cancer; it can be administered via intraperitoneal route or intravenous infusion.

DOX is an extensively used first-line chemotherapeutic agent in patients with malignant breast tumors. However, the use of DOX is also associated with numerous side effects, such as cardiotoxicity, renal, reproductive, and life-threatening hepatic toxicities, mainly because of non-specific targeting and short half-life [2, 3]. Treatment with DOX was reported to raise serum biomarkers of kidney and liver damage in both human patients and animal models [4]. Oxidant production can be increased in respiratory and heart muscles by exposure to DOX, contributing to contractile failure [5]. Pre-existing comorbidities, like immunosuppression, nutritional deficiency, and hepatitis viruses, results in the host's vulnerability to DOX-triggered liver damage [6].

Aiming to improve the efficacy and decrease the toxicity of chemotherapy, a large number of nanocarrier platforms has been proposed as smart drug-delivery systems (DDS). The use of nanocarriers in cancer treatment enables selective delivery of anticancer drugs to the tumor region, and the concentration of the drug can be decreased in healthy tissues [7-21]. In this way, drug effectiveness can be dramatically enhanced while minimizing systemic adverse toxic effects [22-26]. Among the nanotechnological platforms, microemulsions are considered the most versatile and fascinating strategy to enhance the stability and solubility of chemotherapeutic hydrophobic drugs, improve in vivo parameters, and increase cell uptake [27, 28]. The distribution of the incorporated therapeutic drugs can be beneficially altered [29, 30]. In an

earlier report, DOX-loaded microemulsions were prepared that presented interesting colloidal properties. The micellar formulation was composed of polyethylene glycol, vitamin E, and poloxamine Tetronic® T1107. Specifically, this newly prepared micellar system showed a marked cytotoxic effects against breast cancer cells and also appeared highly cytotoxic for ovarian tumor cell line (SKOV-3) as compared to the commercial product DOXil® as well as free DOX. Moreover, it exhibited markedly increased in-vitro cell uptake against MDA-MB-231 and SKOV-3 cells than free DOX and DOXil® counterparts [31]

[32] comparatively assessed the in vivo toxicity of DOXil®, free DOX, and DOX-loaded micelle nanoformulation in a Danio rerio model. Experimental findings revealed that the DOX-loaded nanomicellar system-induced minimal morphological alterations, low cardiotoxicity, and reduced neurotoxicity as compared to the other DOX-based options. Recently, Cagel et al. [33] observed significantly higher in vitro cytotoxic properties of DOX-incorporated micelles than DOXil® in 4T1 murine breast tumor cell line. In contrast to free DOX and liposomes, the intracellular level of DOX for the DOX micellar system was markedly augmented in these cells. Besides, DOX-loaded micelles also induced significantly less cardiac injury and better in vivo antitumor effects than DOXil®, while exhibiting equal effectiveness to the free drug.

Microemulsion-mediated DDSs have gained increasing scientific interest owing to their high aqueous solubilization, reticuloendothelial system (RES) escaping ability, and passive tumors targeting via EPR mechanism [34-36]. Polymeric micelles with nanoscopic unique core-shell architectures and particle diameter below 100 nm display a set of attractive features, such as the ability to encapsulate both hydrophobic and hydrophilic antitumor drug molecules, sustained drug loading efficacy, extended circulation duration, and ability to functionalize with various targeting modifiers [37-39]. Many reports have demonstrated the encapsulation of therapeutic substances or drugs in Pluronic micelles to enhance their efficacies for various cancer treatments. For example, silibinin-incorporated Pluronic F68 microemulsions displayed augmented stability, increased water solubility and bioavailability, and sustained release of the loaded drug compared to pure silibinin [40]. Likewise, the Pluronic P123 micellar system could also efficiently load docetaxel drug with sustained *in vitro* cytotoxic effects on HepG2 cell lines [41]. Pluronic F68 system bearing paclitaxel was formulated as a multipurpose drug transport

carrier to treat hepatocellular cancer. Results revealed that the synthesized nanotherapeutic system yielded significant inhibition of angiogenesis and cell propagation [42].

Our research group recently developed Pluronic F127-based biodegradable and biocompatible microemulsions to increase the solubility and delivery of tocopherol. Besides exhibiting high encapsulation capacity, the as-formulated microemulsion-based nanocarriers presented a sustained release profile of the cargo [43]. Nonetheless, literature is lacking in the preparation of oil-in-water F127 microemulsions for cancer treatment. The present work aims to evaluate i*n vitro* cytotoxic effects of DOX-loaded Pluronic F127 microemulsions in comparison with standard DOX in breast and oral carcinoma cancer cell lines, which has not been explored to date. Since this newly developed formulation exerted favorable *in vitro* anticancer effects, preliminary in vivo studies (rat model) were also carried out in order to confirm the suitability of these nanocarriers for new cancer treatment strategies.

2. Materials & Methods

2.1 Materials for microemulsions

Standard laboratory grade chemicals, including DOX, sodium caprylate, and ethyl butyrate, were provided by Sigma Chemical Co. Pluronic surfactant F127 was procured from BASF Inc. (Mount Olive, NJ)

2.2 Formulation of DOX-incorporated microemulsions

The synthesis of DOX-incorporated oil-in-water microemulsions [44] were prepared as 1% w/w solutions of ethyl butyrate by the vigorous stirring of a suitable amount of sodium caprylate, PBS (pH = 7.4), and at a fixed oil-to-surfactant molar ratio (Ow = [ethyl butyrate]/[F127] = 1). Scheme A portrays the schematic representation of the newly synthesized Pluronic microemulsion structure and contents.

Scheme A. Schematic of Pluronic microemulsions.

2.3 Computational study

DFT calculations using the M06-2X [45] functional along with the 6-31G* basis set was used to optimize the geometry of monomers and the studied complexes. The M06-2X functional is a dispersion corrected hybrid meta exchange-correlation functional [45]. All the calculations were

done with the Gaussian 03 program package [46]. The aqueous solvation effect was included in the geometry optimization using the self-consistent reaction field (SCRF) method and the polarizable continuum model (PCM) [47, 48]. The interaction energy (E_{int}) values can be calculated as follows:

$$E_{int} = E_{complex} - E_{monomer_1} - E_{monomer_2} + E_{BSSE} \quad (1)$$

Where $E_{complex}$ shows the total energy of obtained complexes after interaction of two monomers and the $E_{monomer_1}$ and $E_{monomer_2}$ indicate the total energies of the optimized monomers. The E_{BSSE} is the basis set superposition error, which was calculated using the counterpoise correction scheme outlined by Boys and Bernardi [49]. The natural bond orbital (NBO) method [50, 51] was used to analyze the natural population and the charge transfer during the reaction course. The Bader quantum theory of atoms in molecules was also performed to analyze the topological parameters related to the intermolecular hydrogen bonds formed on the studied complexes [52, 53]. The QTAIM calculations were done with the AIM2000 program [54] at the M06-2X/6-31G* level of theory.

2.4 Characterization of DOX-loaded microemulsions

2.4.1 Digital light scattering (DLS)

DLS characterization of DOX-incorporated microemulsions was carried out using an ALV-5000F Goniometer System coupled with a diode-pumped solid-state laser to supply polarized incident light. The system was also integrated with a digital correlator (ALV SP-86) with a sample range of 25 ns to 100 ms. DLS was performed at an angle of θ = 90° to the incident ray by calibrating the intensity scale by toluene against scattering. Before measuring, the sample solutions were directly filtered into scattering cells using Millipore Millex filters (0.22 μm porosity) and equilibrated for 10 min at the required temperature. In order to acquire a fitted correlation function, the sampling time was 5-10 min. All the experiments were carried out three times.

DLS is a powerful analytical tool to evaluate and characterizing the particle size and diffusion coefficient of nanoparticles in solution. The time-mediated light scattering intensity from the colloidal suspension is a variable quantity dependent on Brownian movement, diameter, and diffusive behavior of nanoscale particles in solution. A digital correlator within the DLS tool

assesses the similarity extent between two signals over a period. If the signal intensity of a specific part of the speckle pattern at a given point is comparable to the signal strength a very short time later, the resulting two signals are very similar, and they strappingly correlate with each other. On the other hand, the correlation between two signals decreases with time due to a decrease in the similarity of two signals by Brownian motion.

The decay rate, Γ, obtained by fitting a single exponential function to the autocorrelation function of samples, is related to the diffusion coefficient by using [43, 55-57]

$D = \Gamma/q^2$ (1)

where q illustrates the scattering vector [43, 55-57]

The diffusion coefficient of nanoparticles or micelles can be characterized as R_h according to the Stokes-Einstein equation [43, 55-57]

$R_h = \frac{k_B T}{6\eta\pi D}$ (2)

where k_B and η denote the Boltzmann constant and water viscosity, respectively.

2.4.2 Entrapment efficiency (EE%) of DOX

The UV-spectrophotometric approach (Agilent Technologies, Cary 50, USA) was used to calculate the content of DOX in the prepared formulations [15, 17, 58]. DOX stock solution (250 µg/mL) was diluted with ethanol/PBS 7.4 (1:1) from the DOX product drug. At wavelengths of 200 to 700 nm, the absorbance peak of DOX was initially determined. The DOX showed a characteristic peak at a wavelength of 480 nm. For calibration curves, DOX working standard solutions were prepared by diluting the stock solution within a concentration range of 250-2 µg/mL, and spectrophotometric determination was carried out at 480 nm. The curve (R^2 = 0.9902) was found to be linear and reproducible. DOX-containing microemulsions were centrifuged at 20000 rpm for 60 minutes (model MC-20000, Medline, United Kingdom). Supernatant content was calculated by absorbance read at 480 nm. Eventually, EE% was determined based on total microemulsion DOX content (250 µg/mL) (below equation):

$$Entrapment\ effeciency\% = \frac{(Total\ Dox - Free\ Dox)}{Total\ Dox} \times 100 \quad (1)$$

2.4.3 Release study

Release activity was tested using a dialysis technique with a 6000 Dalton pore size dialysis membrane [15, 58, 59]. For at least 12 hours before use, the dialysis bag was immersed in the

PBS buffer as a receptor. As a donor portion, 1 mL of DOX solution or DOX-loaded microemulsion was placed in the dialysis bag. Also, in the receiver chamber, 50 mL of PBS 7.4/ethanol was added. DOX release tests at 37 °C and speed of 90 rpm were conducted over 24 hours. 1 mL of the buffer medium as the receiver was collected at various time intervals. Subsequently, the same quantity of fresh buffer (preheated at 37 ° C before replacement) was added to the receiver to maintain a steady volume. The UV spectrophotometer measured the absorbance of the samples at a 480 nm wavelength. The released DOX was withdrawn and analyzed in a quartz cuvette with an area of 1 cm^2 using a UV-Vis spectrophotometer (Agilent Technologies, Cary 100, USA). Each experiment was performed in triplicate.

The release of DOX was evaluated by fitting zero-order, first-order, Higuchi, and Korsmeyer-Peppas models according to research by Rahdar *et al.*, 2019. The profile of DOX release (%) against time for the zero-order, profile of log of the % release against time for the first-order, profile of the % release against the square root of time for the Higuchi, and profile of log of the % release against the log of time for Korsmeyer-Peppas was plotted.

2.5. In vitro experiments

2.5.1 Chemicals

Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and amphotericin B were purchased from Sigma-Aldrich Co (Steinheim am Albuch, Germany). Plastic materials were procured from Sorfa (Sorfa Life Science Research Co., Ltd., Zhejiang, China). The phosphate buffered saline (PBS), 1% penicillin/streptomycin solution, and RPMI 1640 and Dulbecco's modified Eagle's medium (DMEM) culture mediums were supplied by INOCLON (G. Innovative Biotech Co, Tehran, Iran). Fetal bovine serum (FBS) was from Biochrome (Berlin, Germany). Other materials were of analytical grade.

2.5.2. Cell lines and culture conditions

MCF7 human breast cancer cells (cultivated in RPMI 1640 medium), C152 human oral squamous carcinoma cells, and human umbilical vein endothelial cells (HUVECs) (both cultivated in DMEM medium) were provided by the National Cell Bank of Iran, Pasteur Institute of Iran. Culture medium was supplemented with 10% heat deactivated FBS, 50 U/mL of

penicillin, 50 µg/mL of streptomycin, and 250 µg/mL of amphotericin B. Cells were kept at 37°C in a 5% CO_2-95% air humidified atmosphere.

2.4.3. Cytotoxicity effects of standard free DOX and DOX microemulsions

The cytotoxic effects of free DOX and DOX microemulsions were examined using the MTT colorimetric assay (Gerlier and Thomasset, 1986). Cells (5×10^4 cell/mL) were cultured in 96-well microplates. After 24 h, the culture medium was replaced with the one containing DOX in standard or encapsulated form at 0.05 to 0.8 µg/mL. After 48 h, the supernatant was carefully discarded and replaced with MTT solution (0.5 mg/mL) and kept at 37°C for 3.5 h. Later, the MTT was removed and replaced with DMSO for complete solubilization of formazan crystals. The absorbance of dissolved formazan crystals was read at 570 nm using a microplate reader. Cell viability was calculated by the following formula:

Cell viability (%) = OD sample / OD control × 100.

The half-maximal inhibitory concentration (IC_{50}) of standard DOX and F127/DOX microemulsions was calculated using GraphPad Prism software version 7.0.

2.5.4. Morphological evaluation

MCF7, C152, and HUVEC cells (2×10^4 cell/well) were seeded in 24-well plates and incubated for 24 h. The next day, cells were treated with increasing concentrations of standard and encapsulated DOX (from 0.05 to 0.8 µg/mL), and untreated cells served as control. After 48 h, cell morphology changes were monitored using an inverted phase-contrast microscope (IX71, Olympus Inc.) and imaged with a digital camera.

2.5.5. LDH-based cytotoxicity assay

The LDH leakage was assessed in the medium of cultivated cells using a colorimetric lactate dehydrogenase (LDH) cytotoxicity assay kit (KLDH96, Kalazist Co., IRAN) following the manufacturer's protocol. Cells (300×10^5/well) were cultivated in a 6-well microplate and incubated for 24 h. Next, cells were exposed to standard DOX and F127/DOX microemulsions (with IC_{50} concentrations) and kept in an incubator for another 48 h. Then, 100 µL of the supernatant was placed into a 96-well microplate for analysis. The percentage of LDH leakage was calculated following the formula:

(OD test – OD blank)/(OD positive − OD blank),

where OD test represents the optical density (OD) of untreated cells, OD positive represents the OD of the positive control cells or cells treated with standard DOX and 127/F127/DOX microemulsions (with concentrations equal to their IC_{50} values), and OD blank is the OD of the wells containing no cells. The absorption was read at 540 nm against 592 nm as a background.

2.6. In vivo experiments

2.6.1. Design

For the in vivo study, male adult white Wistar rats (mean weight of 269 g) were used. Rats were kept in standard conditions and in a circadian rhythm of 12 h of light and 12 h of darkness. Rats had free access to standard laboratory rodent chow and tap water. The injection and sampling procedure was conducted following ethical guidelines of the Animal Ethics Committee of the Veterinary Medicine Faculty, University of Zabol (Zabol, Iran) and also with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health (NIH) publication 86–23; revised 1985.

In the current experimental study, thirty-two male adult Wistar rats (mean weight 223 ±15 g) were used. Rats were obtained from the animal breeding colonies of the laboratory animal house of the University of Zabol, Zabol, Iran. Before performing the experiments, rats were housed in the experimental room to get used to the situation. Control rats were treated with normal saline intraperitoneally, while the rats of the second group were treated with bulk doxorubicin at 12 a dose of mg/kg. The third and fourth groups received doxorubicin microemulsions at 12 mg/kg and 24 mg/kg doses, respectively.

2.6.2. Determination of biochemical parameters

At the end of the study, blood samples were collected from the heart of rats and were sent to the clinical pathology laboratory. Blood samples were then immediately centrifuged at 3000 × g for 15 minutes at 4 °C, and serum was stored at −80 °C until use. Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), and creatinine were measured by Pars Azemoon reagent kits. (Pars Azmoon Company, Tehran, Iran). Hepatic malondialdehyde (MDA) content was measured by Ohkawa method with minor modifications [60]

2.6.3. Histological examination

In order to investigate the histopathological changes, after blood collection, rats were euthanized by using the cervical dislocation method, and kidney and liver slices were put into 10% neutral formalin. After routine histopathological preparation techniques, liver and kidney sections were investigated by a light microscope (Olympus, Tokyo, Japan) at 40 magnifications to detect histological changes.

2.6.4. Statistical analysis

The collected biochemical data were statistically analyzed using the IBM-SPSS software (version 20), and the significance level was set at 5%. In order to investigate the normality of data distribution, we used Kolmogorov-Smirnov test. The one-way analysis of variance (ANOVA) and Tukey's post-hoc test was performed to evaluate the statistical significance between the groups.

3. Results and Discussion

3.1. Synthesis and characterization of DOX-loaded microemulsions

The Pluronic polymer was amalgamated with DEF solution (surfactant phase) followed by dissolving in fatty acids and ethyl butyrate ("oil" phase) for the preparation of DOX-loaded microemulsions. Fig. 1a shows the autocorrelation function (ACF) against time for as-prepared microemulsions. Fitting a curve to the ACF yielded a decay rate value [43, 55-57]. The hydrodynamic diameter of DOX-based microemulsions was measured to be approximately 7 nm from the experimental data ($3.1938 \cdot 10^{-11}$ m^2/s) (Fig. 1a).

Figure 1. A) DLS autocorrelation function of microemulsions, B) Digital photograph of the microemulsion solution after standing for 3 months.

3.2 Quantum mechanics calculations

Interactions of DOX with ethyl butyrate (DOXE), one unit of the polymeric chain of surfactant F127 (DOXF), and sodium caprylate (DOXS) were studied. The structure optimization of the pristine monomers and obtained complexes was performed using M06-2X functional. Figure 2 shows the optimized structure of studied monomers.

Figure 2: The optimized structures of compounds studied in the present work.

All possible positions for interaction between the mentioned monomers were considered. Figure 3 shows the adsorption energy values and also, indicates the initial and optimized structures of complexes.

Figure 3: The initial (up) and optimized (down) structures of the different studied configurations and the related interaction energy (in kJ/mol).

Figure 3 shows that the DOXF1, DOXF2, and DOXF3 complexes are the most stable complexes belonging to different positions of F127 relative to the DOX. NBO analysis provides an effective method for investigating intermolecular interactions and a suitable basis for studying charge transfer within the molecules. The second-order perturbation energy ($E^{(2)}$) values obtained from the NBO analysis are shown in Table 1.

Table 1. The NBO analysis of all studied configurations.

DOXF1	**$Lp_{(O94)} \rightarrow \sigma^*_{(C4\text{-}H43)}$**
	1.70
DOXF2	$Lp_{(O77)} \rightarrow \sigma^*_{(O23\text{-}H54)}$
	0.92
DOXF3	$\sigma_{(O94\text{-}H95)} \rightarrow$

	$\sigma^*_{(C1\text{-}O2)}$
	0.13
DOXS1	$\sigma_{(C72\text{-}H84)} \rightarrow \sigma^*_{(C16\text{-}H47)}$
	0.72
DOXS2	$Lp_{(O78)} \rightarrow \sigma^*_{(C1\text{-}H42)}$
	1.37
DOXS3	$Lp_{(O77)} \rightarrow \sigma^*_{(C29\text{-}H62)}$
	1.51
DOXE1	$\sigma_{(C72\text{-}H82)} \rightarrow \pi^*_{(C11\text{-}C12)}$
	0.24
DOXE2	$\sigma_{(C72\text{-}H82)} \rightarrow \sigma^*_{(C24\text{-}H56)}$
	0.20
DOXE3	$\pi_{(C69\text{-}O73)} \rightarrow \sigma^*_{(O27\text{-}H60)}$
	1.50

Close inspection of Table 2 indicates that the electron transfer occurs from the orbitals of ethyl butyrate, F127, and sodium caprylate to the sigma anti-bond orbital of DOX molecule in all studied complexes. It is worth mentioning that the transfer of electrons happens from the proton acceptor to the proton donor in the positions with intermolecular hydrogen bonds.

The charge transfer is known as the electron density migration from the highest occupied molecular orbital of an electron donor to the lowest unoccupied molecular orbital of an electron acceptor from the viewpoint of the molecular orbital theory. In other words, the partial charge transfer will occur from the filled orbital of one fragment to the vacant orbital of another fragment. The HOMO and LUMO energy values of monomers were applied to calculate the

$|HOMO_{(monomer_1)}- LUMO_{(monomer_2)}|$ and the $|HOMO_{(monomer_2)}-LUMO_{(monomer_1)}|$ values and the results were listed in Table 2.

Table 2. The values of $|HOMO_{(monomer_1)}- LUMO_{(monomer_2)}|$ and the $|HOMO_{(monomer_2)}-LUMO_{(monomer_1)}|$ energies at M06-2X levels.

|HOMO(DOX)-LUMO(F127)|	**|HOMO(F127)-LUMO(DOX)|**
0.38	0.26
|HOMO(DOX)-LUMO(ethyl butyrate)|	|HOMO(ethyl butyrate)-LUMO(DOX)|
0.33	0.28
|HOMO(DOX)-LUMO(sodium **c**aprylate)|	|HOMO(sodium caprylate)-LUMO(DOX)|
0.27	0.23

These results show that the charge transfer can be performed from the HOMO of F127, ethyl butyrate, and sodium caprylate to the LUMO of DOX. This result is in accordance with the results of the NBO analysis. The energy gap (E_g) and also the HOMO and LUMO orbitals for the DOXF1 complex is shown in Figure 4.

Figure 4. The HOMO and LUMO orbitals and the energy gap of the DOXF1 complex at M06-2X/6-31G* method.

Close inspection of Figure 7 shows that the HOMO is positioned on the F127, and the LUMO orbital is distributed on the DOX molecule in the complex. This result illustrates that the electron density transfer has happened from the HOMO of F127 to the LUMO of DOX.

The strength of hydrogen bond (H-bond) interactions can be determined using the electron density, ρ(r), at the bond critical points (BCPs) [61, 62]. The QTAM method has been widely applied to investigate the ρ values leading to possible H-bonds. The electron density, ρ(r), Laplacian of electron density, $\nabla^2\rho(r)$, and total energy density, H(r), at the BCPs of all complexes are presented in Table 3.

Table 3. The selected topological parameters of investigated complexes (in a.u.) and the energies of the intermolecular hydrogen bond (E^*_{HB} in $kJmol^{-1}$) for all studied complexes, calculated at the M06-2X/6-31G* level.

DOXF1	**ρH_{76}-N_{25}**	**$\nabla^2\rho H_{76}$-O_{75}**	**H**	**E^*_{HB}**	**DOXS1**	**ρH_{80}-O_{36}**	**$\nabla^2\rho H_{80}$-O_{36}**	**H**	**E^*_{HB}**	**DOXS3**	**ρH_{89}-N_{25}**	**$\nabla^2\rho H_{89}$-N_{25}**	**H**	**E^*_{HB}**	**DOXE2**	**ρH_{87}-O_{10}**	**$\nabla^2\rho H_{87}$-O_{10}**	**H**	**E^*_{HB}**
	0.0429	0.1130	-0.0057	-51.9967		0.0061	0.0243	0.0014	-4.1982		0.0040	0.0142	0.0009	-2.3064		0.0074	0.0300	0.0017	-5.3968
	ρH_{85}-O_2	$\nabla^2\rho H_{85}$-O_2	H	E^*_{HB}		ρH_{84}-O_{36}	$\nabla^2\rho H_{84}$-O_{36}	H	E^*_{HB}		ρH_{82}-O_{27}	$\nabla^2\rho H_{82}$-O_{27}	H	E^*_{HB}		ρH_{87}-O_{13}	$\nabla^2\rho H_{87}$-O_{13}	H	E^*_{HB}
	0.0094	0.0356	0.0018	-7.0718		0.0054	0.0212	0.0013	-3.5735		0.0103	0.0388	0.0018	-7.9027		0.0066	0.0259	0.0015	-4.6040
	ρH_{89}-O_2	$\nabla^2\rho H_{89}$-O_2	H	E^*_{HB}		ρH_{85}-O_{32}	$\nabla^2\rho H_{85}$-O_{32}	H	E^*_{HB}		ρH_{60}-O_{77}	$\nabla^2\rho H_{60}$-O_{77}	H	E^*_{HB}		ρH_{77}-N_{25}	$\nabla^2\rho H_{77}$-N_{25}	H	E^*_{HB}
	0.0133	0.0505	0.0021	-11.1503		0.0027	0.0112	0.0008	-1.6199		0.0466	0.1470	-0.0051	-61.6410		0.0147	0.0416	0.0009	-11.1701
	ρH_{93}-O_2	$\nabla^2\rho H_{93}$-O_2	H	E^*_{HB}		ρH_{89}-O_{32}	$\nabla^2\rho H_{89}$-O_{32}	H	E^*_{HB}		ρH_{62}-O_{77}	$\nabla^2\rho H_{62}$-O_{77}	H	E^*_{HB}		ρH_{83}-N_{25}	$\nabla^2\rho H_{83}$-N_{25}	H	E^*_{HB}
	0.0077	0.0308	0.0017	-5.6489		0.0060	0.0215	0.0012	-3.8449		0.0111	0.0378	0.0016	-8.2887		0.0047	0.0168	0.0010	-2.8405
DOXF2	ρH_{76}-O_{13}	$\nabla^2\rho H_{76}$-O_{13}	H	E^*_{HB}	**DOXS2**	ρH_{90}-O_{32}	$\nabla^2\rho H_{90}$-O_{32}	H	E^*_{HB}	**DOXE1**	ρH_{84}-O_2	$\nabla^2\rho H_{84}$-O_2	H	E^*_{HB}	**DOXE3**	ρH_{61}-O_{73}	$\nabla^2\rho H_{61}$-O_{73}	H	E^*_{HB}
	0.0194	0.0753	0.0022	-18.8185		0.0085	0.0300	0.0015	-5.9679		0.0091	0.0343	0.0017	-6.7485		0.0083	0.0313	0.0016	-5.9940
	ρH_{76}-O_{13}	$\nabla^2\rho H_{76}$-O_{13}	H	E^*_{HB}		ρH_{91}-O_{13}	$\nabla^2\rho H_{91}$-O_{13}	H	E^*_{HB}		ρH_{78}-O_{10}	$\nabla^2\rho H_{78}$-O_{10}	H	E^*_{HB}		ρH_{60}-O_{73}	$\nabla^2\rho H_{60}$-O_{73}	H	E^*_{HB}
	0.0194	0.0753	0.0022	-18.8185		0.0082	0.0280	0.0014	-5.5827		0.0062	0.0226	0.0013	-4.0488		0.0259	0.0901	0.0010	-26.9214
	ρH_{54}-O_{77}	$\nabla^2\rho H_{54}$-O_{77}	H	E^*_{HB}		ρH_{87}-O_{13}	$\nabla^2\rho H_{87}$-O_{13}	H	E^*_{HB}		ρH_{82}-O_{13}	$\nabla^2\rho H_{82}$-O_{13}	H	E^*_{HB}		ρH_{85}-O_{27}	$\nabla^2\rho H_{85}$-O_{27}	H	E^*_{HB}
	0.0077	0.0285	0.0015	-5.4033		0.0055	0.0227	0.0014	-3.7867		0.0108	0.0373	0.0016	-8.0623		0.0101	0.0362	0.0017	-7.5394
DOXF3	ρH_{95}-O_{10}	$\nabla^2\rho H_{95}$-O_{10}	H	E^*_{HB}		ρH_{87}-O_{10}	$\nabla^2\rho H_{87}$-O_{10}	H	E^*_{HB}										
	0.0119	0.0493	0.0023	-10.0394		0.0087	0.0302	0.0015	-6.1006										
	ρH_{93}-O_{13}	$\nabla^2\rho H_{93}$-O_{13}	H	E^*_{HB}		ρH_{42}-O_{78}	$\nabla^2\rho H_{42}$-O_{78}	H	E^*_{HB}										

	0.0016	0.0073	0.0005	-0.9648		0.0099	0.0318	0.0013	-6.8952									

The molecular graph demonstrates distinctly the bond critical point, ring critical point (red and yellow balls, respectively), and the bond paths. Figure 5 indicates the molecular graph and contour map of the most stable complex (DOXF1).

Figure 5. A: Molecular graph and **B:** contour map of the DOXF1 complex obtained from the DFT calculation.

The Espinosa method was used to calculate the intermolecular H-bond energy [63]. In the Espinosa method, the individual hydrogen bond energies (E^*_{HB}) were estimated using the ρ(r) values at the hydrogen bond critical points. The maximum ρ(r) and E^*_{HB} values at the H-bond critical points belong to DOXF1 and DOXS3 complexes at the H_{76}-N_{25}, H_{60}-O_{77} bonds, respectively (see Table 3). It can be said that the geometrical parameters are good descriptors to indicate the strength of the H-bond because the obtained results show that there is a good correlation between the E^*_{HB} values and the geometrical parameters. It is found that the shorter the H⋯ Y (Y is the proton acceptor) distance, the stronger the H-bond strength. Therefore, it is reasonable that the H_{76}-N_{25} distance in the DOXF1 complex and the H_{60}-O_{77} in the DOXS3 complex have a minimum bond length (see Figure 6).

Figure 6: The distances between the H with O and N atoms at intermolecular hydrogen bonding sites for **A:** DOXF1 and **B:** DOXS3 complexes.

The $\nabla^2\rho(r) > 0$ and $H(r) > 0$ at the contact points show that the weak H-bonds between the studied monomers except in H_{76}-N_{25} contact of DOXF1 complex and H_{60}-O_{77} bond in DOXS3 that the positive $\nabla^2\rho(r)$ and the negative H(r) value indicate the medium-strength intermolecular interaction with partially covalent nature in these points. The ρ(r) and $\nabla^2\rho(r)$ values at the bond critical point of X⋯H contact are well correlated with the E^*_{HB} value; as a result, higher values of ρ(r) and $\nabla^2\rho(r)$ are related to stronger hydrogen bond interactions. The correlation between ρ(r) and $\nabla^2\rho(r)$ with E^*_{HB} values are indicated in Figure 7.

Figure 7. Correlation between the calculated **A:** ρ(r) and **B:** $\nabla^2\rho(r)$ with the E^*_{HB} energies.

The thermodynamic parameters, including Gibbs free energy (ΔG), enthalpy (ΔH), and entropy (ΔS) of all complexes, were calculated (see Table 4).

Table 4. Thermodynamic parameters (all in kJ/mol) of all studied complexes.

Complexes	ΔH	ΔG	TΔS
DOXF1	-54.794	10.163	-64.957
DOXF2	-44.799	11.754	-56.553
DOXF3	-42.147	14.349	-56.496
DOXS1	-18.515	29.190	-47.705
DOXS2	-30.768	21.117	-51.886
DOXS3	-23.123	23.991	-47.114
DOXE1	-33.522	19.213	-52.736
DOXE2	-16.997	34.286	-51.284
DOXE3	-30.104	18.602	-48.706

The value of TΔS indicates the entropy changes during the formation process of complexes. The high $T\Delta S < 0$ values determine the $\Delta G > 0$. In other words, the formation process of complexes is thermodynamically unfavorable; therefore, the probability of complex formation is controlled by the entropic factor (It means that $T\Delta S > \Delta H$); therefore, the process needs the larger entropy than the energy changes. The complexes with lower ΔG values are relatively more stable, whereas those with higher Gibbs energy of formation are unstable. Therefore, DOXF1, DOXF2, and DOXF3 complexes with lower ΔG values are more stable than the other complexes. The negative enthalpy values display that the interaction between the mentioned monomers is exothermic and enthalpically favorable.

3.3 Entrapment efficiency

One of the significant physicochemical properties in designing drug nanostructures is encapsulation efficiency (EE) [56, 64-66]. High EE guarantees adequate treatment efficacy of

loaded substances at a lower dose than that needed for free chemical molecules to be administered, thus minimizing the level of adverse side effects [59, 64, 67]. The DOX microemulsion encapsulating percentage was 94±1.5 %. The interactions between DOX and microemulsions, which can lead to a more rigid microemulsion membrane and affect the release of DOX, can lead to this high EE percentage. The microemulsion core is also hydrophobic, and it could be concluded that DOX was mainly stuck in the core of microemulsion.
In a similar study, Yung-Chih et al. prepared Pluronic F127 stabilized microemulsion and mentioned that EE% of etoposide (ETO), carmustine (BCNU), and DOX in PEPC1 NPs ($MPEG_{114}$-PCL_{40}) and PEPC2 NPs ($MPEG_{114}$-PCL_{84}) depended on the hydrophobicity and chain length of PCL [68].

3.4 In-vitro release experiment

The DOX solution (free DOX) and DOX-loaded microemulsion in vitro release experiments were performed using dialysis methods at PBS 7.4/ethanol (1:1) and 37 °C. The free DOX release rate was significantly faster than that of DOX-loaded microemulsion, as shown in Figure 8. DOX release percentage reached only 68 % after 24 h for DOX-loaded microemulsion, which was able to allow a slow release rate.

Figure 8. *In vitro* release of free DOX and DOX-loaded microemulsion at PBS 7.4/ethanol (1:1) and 37°C after 24 hours.

By matching the first-order, zero-order, Higuchi, and Korsmeyer-Peppas model, DOX release kinetics were evaluated. As shown in Figure 9, the Korsmeyer-Peppas model was better suited to the microemulsion DOX release rate based on the R^2 (determination coefficient) value. This kind of release is related to the processes of microemulsion swelling and DOX diffusion. At the beginning of the process and in the presence of water, the microemulsion may gradually expand, allowing the loaded drug to spread through the membrane. The lipophilic fatty cores, however, will decrease the diffusion coefficient of DOX. The value of n=0.48 also indicates that the mechanism of DOX release is quasi-Fickian, so microemulsion diffusion will play a key role in this nanocarrier [68].

The effect of different parameters on the release of active ingredients from nanocarriers has been evaluated in several studies [69-71]. For example, Yung-Chih et al. prepared Pluronic F127 stabilized microemulsions and evaluated cumulative releases of different drugs from PEPC1 NPs and PEPC2 NPs at various pH levels [68]. In medium containing DPBS at pH 7 and pH 5, the average release rates of ETO from PEPC1 NPs were 31.8 % and 56.0 %, respectively, while those from PEPC2 NPs at pH 7 and pH 5 were 14.5 and 40.1 %, respectively. For DOX and BCNU, similar trends were obtained. The authors of this study believed that PEPC2 NPs could have greater hydrophobicity, leading to a lower capacity to release drugs than PEPC1 NPs.

Figure 9. Profiles of different kinetic mode for release of DOX from microemulsion.

3.5. Cytotoxicity evaluation and morphological alterations

Cell killing effects of standard DOX and F127/DOX microemulsions in malignant and non-malignant cell lines were assessed by MTT colorimetric assay (Figure 10). Compared with untreated cells, standard DOX significantly diminished the viability of MCF7 and C152 cells following a concentration- and time-dependent trend ($P < 0.05$). The same trend, but to a greater extent, was observed when different cells were exposed to increasing concentrations of F127/DOX microemulsions. IC_{50} values for 48 h treatment of HUVEC, C152, and MCF7 cells with standard DOX were 0.724, 0.189, and 0.799 μg/mL, while the IC_{50} values of F127/DOX microemulsions were 0.113 μg/mL (for HUVEC cells), 0.011 μg/mL (for C152 cells), and 0.045 μg/mL (for MCF7 cells). Among the studied cell lines, C152 cells were most sensitive to F127/DOX microemulsions.

Figure 10. Cytotoxicity evaluation of DOX and F127/F127/DOX microemulsions on HUVEC (non-malignant), MCF7, and C152 (malignant) cells after 48 h treatment. (**$P < 0.05$ compared with untreated cells)

Compared with standard DOX, F127/DOX microemulsions markedly induced cell death in MCF7 and C152 cells (lower IC_{50} values). This indicates the potent cell-killing effects of our newly synthesized microemulsions against cancer cells. However, this might not be desirable, as we also concurrently observed an evident reduction in the number of HUVEC viable cells exposed to F127/DOX microemulsions. Besides a mild to moderate decrease in the number of viable cells, treatment with standard DOX at increasing concentrations caused no evident

morphologic alterations in HUVEC (Figure 5) and MCF7 (Figure 6) cells. C152 cells were rounded up, shrunk, and apoptotic bodies were formed with increasing concentrations of standard DOX at 48 h (Figure 10). While exposing HUVEC cells to high concentrations of F127/DOX microemulsions (0.1 μg/mL and more), the number of rounded cells was increased, and progressive nuclear shrinkage and apoptotic bodies were observed (Figure 11). Phase-contrast microscopy of MCF7 and C152 cells revealed the same substantial concentration-dependent morphological alterations (Figures 12 and 13, respectively). Most importantly, when treated with high concentrations of F127/DOX microemulsions (> 0.4 μg/mL), cells became detached from the culture dish, which was not observed when cells were treated with standard DOX at the same concentrations.

Figure 11. Morphological changes photographed after monitoring of HUVEC cells for 48 h at increasing concentrations of standard DOX and F127/DOX microemulsions. Concentration-dependent cytotoxicity was observed. Cells were rounded up and shrunk when exposed to high concentrations of F127/DOX microemulsions.

Figure 12. Morphological alterations photographed after monitoring of MCF7 cells for 48 h at five different concentrations of standard DOX and F127/DOX microemulsions. F127/F127/DOX microemulsions caused concentration-dependent cytotoxicity and caused substantial cell morphology changes compared with standard DOX.

Figure 13. Morphological assessment of C152 cells treated with increasing concentrations of standard DOX and F127/F127/DOX microemulsions for 48 h. F127/F127/DOX microemulsions mediated a concentration-dependent cell-killing effect and markedly increased the number of rounded cells and apoptotic bodies.

In 2009, Sardão et al. reported that free DOX could induce morphological changes on the nuclei and mitochondria of the H9c2 myoblast cell line [72]. Moreover, several studies reported the desirable anticancer potential of DOX-loaded nanocarriers. In 2019, Abbasian and coworkers prepared LA/chitosan/NaX/Fe_3O_4/DOX nanofibers as an anticancer DDS and examined their

cytotoxic effects against carcinoma cells. Based on their observations, these nanofibers decreased the viability of H1355 cancer cells by 82% after 7 days of treatment [73]. Li et al. proposed that Pluronic F127 microemulsions could serve as useful alternative vehicles for targeted delivery of drugs [74]. Chen and colleagues synthesized F127-modified nanosheets (MoOX@F127) with an ultra-high DOX loading efficiency. They found synergistic anticancer effects when combining photothermal therapy with chemotherapy using DOX-loaded nanosheets [75]. In line with these findings (although the DOX carrier was different), we found enhanced antitumor drug efficiency for F127/DOX microemulsions compared to standard DOX in malignant cells, which might be the consequence of the controlled release of DOX by use of this novel formulation. This further confirms that Pluronic F127 can actually be utilized as an advantageous vehicle for carrying hydrophobic anticancer drugs to perform local cancer treatment, as suggested elsewhere [76].

3.6. LDH release assay

Compared to untreated cells, treatment with standard DOX and F127/DOX microemulsions increased the LDH leakage by 2.73 and 2.89 (for HUVEC cells), 2.86 and 5.11 (for MCF7 cells), 2.40 and 3.53 (for C152 cells) folds, respectively (Figure 14). This indicates that both standard DOX and F127/DOX microemulsions induced remarkable necrotic damage to these cells and substantially enhanced the release of intracellular LDH ($P < 0.05$). The amount of LDH released by standard DOX and F127/DOX microemulsions were not statistically different ($P > 0.05$). However, compared to standard DOX, F127/DOX microemulsions significantly segmented the leakage of cytosolic LDH in MCF7 and C152 cells ($P < 0.05$).

Previous studies indicated that apoptosis might be the primary mechanism of DOX-induced cell death [77]. Lately, it has been established that DOX-induced necrosis is mediated by poly-ADP-ribose polymerase 1 [78] or Bcl-2-like 19kDa-interacting protein 3 [79]. In our study, we treated malignant cells with F127/DOX microemulsions and observed some typical morphological features that are considered as the hallmarks of apoptosis, such as cell roundness, shrinkage, and formation of membrane-bound apoptotic bodies. Furthermore, F127/DOX microemulsions dramatically enhanced LDH leakage, indicating the induction of necrosis or late-apoptosis in these cells. Altogether, our results suggest that our microemulsions might kill cancer cells triggering both apoptosis and necrosis pathways. More *in vitro* investigations should be carried

out to better elucidate the underlying mechanisms by which these microemulsions induce cell death.

Figure 14. The percentage of LDH leakage from different cells treated with standard DOX and F127/F127/DOX microemulsions for 48 h. (**$P<0.05$ compared with untreated cells)

3.7 Biochemical analysis

As shown in Table 5, the intraperitoneal injections of F127/DOX microemulsions at a dose of 6 mg/kg had no statistically significant effect on serum AST and serum ALT (P> 0.05). In contrast, treatment with the bulk (free) DOX (12 mg/kg) significantly increased serum liver enzymes compared to the control group. Treatment with the 12 mg/kg dose of bulk DOX significantly increased serum BUN and creatinine levels compared to the BUN and creatinine levels of healthy control rats (P <0.05). The intraperitoneal injection of the 12 mg/kg dose of F127/DOX microemulsions had no statistically significant effects on serum BUN and creatinine levels of rats (P >0.05).

The group treated with the 24 mg/kg dose of DOX showed significant increases in serum AST, ALT, BUN, and creatinine levels compared to the healthy control rats (P <0.05). The statistical analysis revealed a statistically significant difference in liver MDA content of rats who received the high dose of F127/DOX microemulsions. In contrast, hepatic malondialdehyde content of rats receiving the 12 mg/kg dose F127/DOX microemulsions were not statistically higher than that of the control group.

Table 5. Effects of intraperitoneal injection of DOX microemulsions on serum biochemical parameters and liver malondialdehyde levels.

Item[1]	**Control**	**Treatment**		
		Doxorubicin 12 mg/kg	**Doxorubicin microemulsions 12 mg/kg**	**Doxorubicin microemulsions 24 mg/kg**
AST (U/L)	53.5 ± 7.3	102.8*** ± 22.0	69.6 ± 15.7	93.6*** ± 16

ALT (U/L)	42.3± 9.1	86.5*** ± 15.5	59.5 ± 14.6	72.1**± 14.7
BUN (mg/dl)	17.1 ± 4.8	31.5* ± 2.8	22.1 ± 3.4	29.3*** ± 2.3
Creatinine (mg/dl)	0.78 ± 0.18	1.2** ± 0.18	0.83± 0.11	1.09*± 0.29
Liver MDA (nmol/mg protein)	526.3 ± 78.1	832.5*** ±123.3	611 ± 68.1	731.1** ± 73.9

AST, aspartate aminotransferase; MDA, malondialdehyde; ALT, alanine aminotransferase; BUN, blood urea nitrogen. (*P < 0.05 compared to control group).

3.5. Histopathological examination

The photomicrographs related to the histopathological analysis of rats receiving the bulk form of DOX and F127/DOX microemulsions is shown in Figure 15.

Figure 15.A: Photomicrographs of rat liver tissues of control rats showing normal hepatocytes (H). B: Liver section of a rat received bulk DOX at a dose of 12 mg/kg, arrow shows cytoplasmic fat accumulation, and (H) shows a hepatocyte. C: liver section of a rat treated with F127/DOX microemulsions at a dose of 12 mg/kg, a slight disarrangement of hepatic cords is present, and (H) shows healthy hepatocytes. D: liver micrograph of a rat treated with doxorubicin F127/DOX microemulsions at a dose of 24 mg/kg . Arrow point shows sinusoidal disarrangement. (Alcian blue staining) (Magnification × 40)

Figure 16. A: kidney section of a control rat. B: renal micrograph of a rat received the bulk DOX, arrow shows hyaline cast formation. C: photomicrographs of rat kidney tissues of rats received DOX at a dose of 12 mg/kg, arrow point indicates cytoplasmic vacuolation. D: renal section of a rat treated DOX at a dose of 24 mg/kg. Arrow point shows cytoplasmic vacuolation. Arrow shows bleeding (Hematoxylin and Eosin) (Magnification × 40).

The group receiving the bulk DOX showed severe cytoplasmic ballooning, nuclear pyknosis, and shrunken nucleus (Fig. 15B). Exposure to F127/DOX microemulsions at a dose of 12 mg/kg induced minor histopathological lesions, and only slight sinusoidal disarrangement was observed (Fig. 15C); however, liver sections of rats receiving the 24 mg/kg showed mild hepatocyte ballooning, as observed in (Fig. 15D). There was also slight sinusoidal disarrangement in this group; however, the severity of damage was less prominent compared to the rats receiving the bulk DOX (Fig.15C). The observed cytoplasmic fat accumulation in rats treated with the bulk form of DOX was accompanied by serum biochemical alterations. As shown in Table 1, serum AST and ALT levels were significantly increased in rats treated with the bulk DOX as compared

to the normal control rats. This is consistent with earlier experiments demonstrating that DOX could induce several histopathological and biochemical alterations in the liver [80], kidney [81], and heart [82]. The results of previous studies have shown that DOX has dose-dependent toxicity in different organs, including reproductive organs [83], liver, and heart [84]. Previous studies have also shown that different organs could acutely be injured following exposure to DOX-loaded nanoparticles but could return to normal conditions in a short period according to the studies [85]. The histopathological investigations of renal tissues following intraperitoneal exposure to the dose of 12 mg/kg of free DOX showed severe histological changes (figure 16 B). The histopathological investigations of renal tissues showed dose-dependent renal toxicity, which was in line with previous studies (figure 16 B) [58]. Treatment with the low dose of F127/DOX microemulsions induced slight histopathological changes in renal tissues (figure 16 C). In contrast, the group treated with the high dose (24 mg/kg) of F127/DOX microemulsions showed severe bleeding and cytoplasmic vacuolation (figure 16 D).

These results motivate further investigation of the prepared microemulsions in the context of cancer treatment, also considering that previous in vivo experiments have demonstrated that the microemulsion formulations have better biocompatibility and biodegradability than conventional drug delivery systems. Previous studies have shown that lipid-based nanoformulations of DOX had less systemic toxicity and provided tumor-specific effects as compared with the bulk DOX [86,87]. The antitumor and hepatotoxicity of different DOX nanoformulations have been investigated as molecular nanotherapeutic agents, and DOX encapsulation in micelles or nanoemulsions was reported to increase therapeutic efficacy and antitumor efficiency [86,87].

4. Conclusion

Pluronic F127-based microemulsion (around 7 nm) was synthesized as a new vehicle for DOX administration in cancer treatment. Computational studies showed that the most stable interactions occur between F127 and DOX; specifically, in all interactions, DOX acts as an acceptor, and the electron transfer occurs from the orbitals of ethyl butyrate, F127, and sodium caprylate to the sigma anti-bond orbital of the DOX molecule.

In vitro studies revealed that F127/DOX microemulsions might trigger independent signaling pathways and exerted favorable cytotoxic effects against breast and oral carcinoma cancer cells. Moreover, *in vivo* results indicated that F127/DOX microemulsions have fewer side effects on

normal rat tissues than the bulk form of DOX. More studies should be carried out to elucidate the precise cell death mechanism in cells treated with these microemulsions. These findings show promise in developing new strategies for cancer treatment.

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