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RESEARCH ARTICLE

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New liposomal doxorubicin nanoformulation for osteosarcoma: Drug release kinetic study based on thermo and pH sensitivity

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A novel approach was developed for the preparation of stealth controlled-release liposomal doxorubicin. Various liposomal formulations were prepared by employing both thin film and pH gradient hydration techniques. The optimum formulation contained phospholipid and cholesterol in 1:0.43 molar ratios in the presence of 3% DSPE-mPEG (2000). The liposomal formulation was evaluated by determining mean size of vesicle, encapsulation efficiency, polydispersity index, zeta potentials, carrier's functionalization, and surface morphology. The vesicle size, encapsulation efficiency, polydispersity index, and zeta potentials of purposed formula were 93.61 nm, 82.8%, 0.14, and −23, respectively. Vesicles were round-shaped and smooth-surfaced entities with sharp boundaries. In addition, two colorimetric methods for cytotoxicity assay were compared and the IC_{50} (the half maximal inhibitory concentration) of both methods for encapsulated doxorubicin was determined to be 0.1 μg/ml. The results of kinetic drug release were investigated at several different temperatures and pH levels, which showed that purposed formulation was thermo and pH sensitive.

KEYWORDS
cytotoxicity, drug delivery, liposome characterization, osteosarcoma, release kinetics

1 | INTRODUCTION

Doxorubicin (DOX) is one of the most common antibiotic drugs, which belongs to the anthracycline family, and is used as chemotherapeutic agent to fight against tumors and leukemias.[1] But the efficacy of this anticancer drug is limited by its many toxic side-effects due to its potential exposure to normal cells.[2,3] Increasing its therapeutic efficacy by reducing the toxicity is necessary for its clinical use. Nanotechnology offers the potential to improve drug

solubility and stability in order to prolong drug half-life in plasma, minimize side-effects, and concentrate the drugs at a target site.[4,5] Liposomes are an important class of biodegradable nanocarriers that sufficiently decrease the drug's side-effects and increase its delivery to the cancer site. Liposomes are biliary safe and biodegradable structures that can be prepared mainly using various phospholipids.[6] Liposomes are often composed of natural and synthetic phospholipids such as soya phosphatidylcholine (SPC) and dipalmitoylphosphatidylcholine (DPPC), respectively.[7,8]

Some liposomes are capable of delivering their drug load inside the cell and even inside different cell compartments. Hence, touted benefits for the use of these "stealth" liposome carriers include reduced systemic phagocytosis and a resultant prolonged circulation time, selective agent delivery through the leaky tumor endothelium (an enhanced permeability and retention effect), as well as reduced toxicity profiles.[9,10] Nowadays, many researchers developed smart liposomal formulations for localized drug action (i.e., to localize and maintain the drug activity at its site of action) and to increase its bioavailability for cellular cancer site. Osteosarcoma is the most common histological form of primary bone tumor, and it is prevalent in children and young adults between the ages of 15 and 19 years. Effective treatment moieties to combat this disease are an urgent and currently unmet need, and novel nanotechnology-based cancer therapies delivering drugs in liposomal nanoparticles to primary and in particular metastatic osteosarcoma tumors are likely key to better treatment options in the future.[11] Fang et al.[12] formulated modified long-circulating magnetic doxorubicin-containing liposomes by ammonium sulfate gradients with ethanol injection. The optimum formula contained egg-PC/cholesterol (5:1 molar ratio) and 0.02 g mPEG. Garbuzenko et al.[13] elucidated the effects of various mole percentages of PEG–DSPE, presence of cholesterol, and the degree of PC saturation on liposome formulation.

In 2009, Ta et al.[14] used a chitosan–dipotassium orthophosphate hydrogel for the delivery of doxorubicin in the treatment of osteosarcoma. Susa et al.[15] loaded the doxorubicin in lipid-modified dextran-based polymeric nanoparticulate system to overcome drug resistance in osteosarcoma in 2009. Ubo et al. also prepared magnetic liposomes with incorporated doxorubicin by reverse-phase evaporation method. They studied the effect of these nanoparticles on osteosarcoma. These nanocarriers increased the drug accumulation in tumor cells via P-glycoprotein (P-gp) independent pathway. Results showed increased apoptosis in bone tumor cells in comparison with free drug.[16] Low et al.[17], used hydrophilic d-aspartic acid octapeptide and one to four 11-aminoundecanoic acid (AUA) to construct acid-sensitive doxorubicin conjugate micelles.

However, the high costs of synthesizing targeted liposomes have raised concerns over the adoption of targeted liposomes as cost-effective drug delivery systems. Moreover, in most studies little information and characterization of the doxorubicin-liposome formulation is presented. We postulate that further optimization of the DOX-containing liposomes with regard to DOX loading efficiency and intracellular DOX release profiles by fine-tuning thermo and pH sensitivity for optimal release profiles within the endosomal system of the cancer cell can be achieved, which may lead to more effective osteosarcoma treatment. We acknowledge that this optimal liposomal DOX formula should meet the criteria of an economical and effective nanodrug delivery system. Hence, the aims of this study were as follows:

- to prepare liposomal DOX particles with different hydration methods and formulations;
- to evaluate synthetic as well as natural phospholipids as main components of the liposome structure;
- to evaluate the thermo- and pH-sensitive properties of the prepared nanocarriers using in vitro release kinetic studies;
- to apply two colorimetric methods for cytotoxicity assessment of the synthesized nanocarrier using the osteosarcoma cell line MG-63 as a model system for osteosarcoma.

2 | MATERIALS AND METHODS

2.1 | Materials

2.1.1 | Cell line

Osteosarcoma MG-63 cells were obtained from the Pasteur Institute of Iran (Tehran, Iran).

Human primary (short-term culture; i.e., passage <10) osteoblasts (Hum 63) were obtained from healthy patients undergoing total knee replacement after informed consent. Cells were grown in an incubator (Memmert GmbH Co. KG, Germany) at 37°C under the atmosphere of 5% (v/v) CO_2 and moist air (95% (v/v)) in DMEM medium (Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS). Heat-inactivated FBS, penicillin/streptomycin (PEST), and trypsin stock solutions were supplied by Invitrogen (Carlsbad, CA, USA). Cells grew as single layer in adherent mode. Cells were passaged when they had reached 90%–100% confluent. Cells for all experiments were only used when they were in their exponential growth phase.

2.1.2 | Chemicals

Doxorubicin HCl was purchased from Ebewe Pharma (Austria). The distearoyl phosphoethanolamine, polyethylene glycol (Lipoid PE 18:0/18:0—PEG2000, DSPE-mPEG

2000), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and soya bean phospholipids with 75% phosphatidylcholine (SPC 80) were obtained from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol was purchased from Sigma-Aldrich Co (St. Louis, MO, USA). All other chemicals used in this study were of commercial analytical grade and used without further purification.

2.2 | Methods

To synthesize liposomal DOX with disparity and desired particle size, controlled release and high encapsulation efficiency of different sets of experiments were categorized as:

- comparison of synthetic and natural phospholipids (SPC 80, DPPC) with those synthesized by hydration method (thin film and pH gradient);
- optimization experiments of cholesterol:phospholipid ratios (1:1.5, 1:1, 1:0.67, 1:0.43, 1:0.25, and 1:0) in different DSPE-mPEG (2000) %;
- kinetic release assay at different values of pH and temperature (pH: 4.5, 5.4, and 7.4; temperature: 25, 37, and 42°C as the room temperature, physiological, and endosomal cancer cells sites condition, respectively);
- cytotoxicity evaluation of the optimized formulation on osteosarcoma cell line using two different methods;

All experiments were conducted by varying one of the parameters while all others were kept fixed. All experiments were carried out in triplicate.

2.3 | Preparation of drug-loaded liposomes

Thin film and pH gradient methods were established for the preparation of DOX-loaded liposomes (DOX-liposome). In brief, DPPC, SPC80 and cholesterol in the various mole ratios were dissolved in chloroform that was later evaporated. The present formulation contained 0 or 3% DSPE-mPEG2000. The lipid-formed film was hydrated with 1,300 µl drug solution (thin film method) and ammonium sulphate (pH gradient method) for 60 min at 55°C using rotary instrument (Heidolph, Germany). Multilamellar vesicles (MLVs) were then sonicated for 45 min using microtip probe sonicator (E–Chrom Tech Co, Taiwan) over an ice bath to produce small unilamellar vesicles (SUVs), which were subsequently dialyzed against phosphate-buffered saline (PBS). For the preparation of liposomal DOX by pH gradient method, the DOX was loaded into the blank liposomes for 60 min at 55°C. The final concentration of DOX in liposomal formulation was 500 µg/ml for in vitro study.

2.4 | Encapsulation efficiency of DOX in liposomes

Doxorubicin-loaded liposomes were finally placed into dialysis cellulose membrane tubing (cutoff: 12–14 kDa) to remove un-encapsulated drug. The amount of liposomal encapsulated doxorubicin was analyzed with a UV spectrophotometer (model T80+, PG Instruments, United Kingdom) at 480 nm after lysing the liposomal solutions with isopropanol (99% purity). A standard curve of DOX was plotted at 480 nm to determine the correlation between the concentration of DOX and its absorbance with a dilution series of isopropanol solution of doxorubicin.

The encapsulation efficiencies were calculated as follows:

$$\text{Encapsulation efficiency}\,(\%) = \frac{\textit{The amount of DOX encapsulated within liposome}}{\textit{Total amount of DOX added}} \times 100$$

2.5 | In vitro thermo- and pH-sensitive DOX release assay

The release of doxorubicin from liposomes was monitored by dialysis (MW cutoff = 12 kDa, Sigma, Germany) against PBS for 48 hr at 37 and 42°C temperature and pH 7.4, 5.4, and 4. To calculate the released DOX, dialysis media were collected at different times and immediately replaced with the same volume of fresh PBS. Samples were analyzed using the UV spectrophotometer at 480 nm. According to the total drug concentration of the liposome formulation, percentage of release was calculated at each time interval.

2.6 | Particle size and zeta-potential measurements

Both the liposomal hydrodynamic diameters (particle size) and surface charges (zeta potential) were measured using dynamic laser scattering technique (Zeta-Sizer instrument, DLS, Malvern Zatasizer Nano-ZS, Worcestershire, UK). Scattered light was detected at room temperature at an angle of 90 degrees, and the diluted samples in 1,700 µl of deionized water (0.1 mg/ml) were prepared and immediately measured after preparation. All measurements were carried out four times, and their mean values were calculated. Also, the average polydispersity index (PDI) of the liposomes was determined. Freshly prepared liposomes had a refractive index of 1.330, and viscosity and dielectric constant of 0.89 cP and 78.54, respectively.

2.7 | Scanning electron microscopy (SEM)

Samples were kept on glass plate, and the remaining solution was evaporated. The samples were coated with gold coater for few seconds to make them conductive, followed by evaluation of the surface morphology (roughness, shape, smoothness, and formation of aggregates) using SEM with 100 watt power instrument (model EM3200, KYKY, China).

2.8 | Cryogenic transmission electron microscopy

The internal structure of nanoliposomes was observed by cryogenic transmission electron microscopy (FEI Tecnai 20, type Sphera, Oregon, USA) equipped with a LaB6 filament at 200 kV. A drop of liposomal solution was placed over a 200-mesh Cu-coated TEM grids, and TEM measurement was performed.

2.9 | Fourier transform infrared (FTIR) spectral evaluation

The nanoliposomal functionalization was investigated using FTIR spectrometer (Model 8300, Shimadzu Corporation, Tokyo, Japan) at 4 cm^{-1} resolution in the transmission mode. For preparation, liposomes were separated from liposomal suspension by centrifugation and the excess liquid was evaporated. Samples were mixed with KBr and pressed into a pellet. FTIR spectrum was scanned in the wavelength range of 400–4,000 cm^{-1}.

2.10 | Differential scanning calorimetry (DSC)

The phase transition temperature of liposomes was evaluated using a DSC (Model DSC 823e, METTLER TOLEDO, Greifensee, Switzerland) to investigate the thermosensitivity of liposomes with 5°C/min heating rate and −20 to 150°C for the scanning range.

2.11 | Physical stability examination

To determine the physical stability of liposomal doxorubicin during storage, the change in particle size, PDI, zeta potential, and the residual amount of the drug in vesicle was evaluated at different time periods. The samples of sealed liposomes in a glass vial were kept at 2–8°C for 6 months under light protection. Stability analysis was performed during 14 and 28 days, and 3 and 6 months interval.

2.12 | In vitro cytotoxicity assays

2.12.1 | MTT assay

The MTT cellular cytotoxicity of all studied formulations was assessed using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described previously.[18] To measure the cytotoxicity, MG-63 osteosarcoma cells and primary bone cell were seeded separately (10^4 cells/well) into a 96-well plate for 24 hr. Then, the cells were treated with an equal volume of fresh medium (an equal volume of fresh medium was added) and different concentrations of all combinations of empty liposome, liposomal DOX, and free DOX, performed in a total of four series of tests as follows:

- control (fresh media, 200 μl)
- empty liposomes (20 μl empty liposome + 180 μl fresh media)
- free DOX (10, 5, and 0.1 μg/ml)
- liposomal DOX in various concentrations (10, 5, and 0.1 μg/ml)

The duration of re-incubation was 24 and 72 hr. Then, 20 μl MTT (5 mg/ml) was added into every 96-well plate and incubated for 3 hr. After that, the supernatant was evacuated and 180 μl of DMSO was added for dissolving crystals. Absorption was recorded using EPOCH Microplate Spectrophotometer (synergy HTX, Bio Tek, USA) at 570 nm.

Based on these measurements, IC_{50} doses (the concentrations of active ingredients necessary to inhibit the cell growth by 50%) of all tests were calculated.

2.12.2 | Alamar blue assay

Cytotoxicity of the blank liposomes and the tumor cell inhibition by liposomal DOX were also evaluated by Alamar blue assay.[18] MG-63 osteosarcoma cells and primary bone cell were cultured at a density of 10^4 cells per well into 96-well plates with DMEM medium, supplemented with 10% FBS at 37°C in a 5% CO_2-humidified atmosphere in an incubator for 24 hr.

The medium was then replaced with fresh medium containing the various concentrations of the samples preprepared blank liposomes or liposomal DOX and incubated with the cells. The concentration of blank liposomes, free doxorubicin, and liposomal doxorubicin was varied from 10, 5, and 0.1 μg/ml. After 24 hr or 72 hr, the medium was removed, each well was rinsed with PBS and 250 μl of Alamar blue solution (10% Alamar blue, 80% medium 199 (Gibco), and 10% FBS, v/v) was added and incubated for further 3 hr. A sample of 200 μl of Alamar blue solution was transferred into a fresh 96-well plate, and the plate was read in an automated microplate spectrophotometer (EPOCH Microplate Spectrophotometer-synergy HTX, Bio Tek, USA) at 570 nm (excitation)/600 nm (emission) wavelength.

3 | RESULTS AND DISCUSSION

3.1 | The selection of appropriate formulation

3.1.1 | Effect of phospholipid type and preparation methods

Liposomal formulations were prepared by thin film and pH gradient methods. Table 1 provides the comparison between various types of phospholipids (synthetic and natural) and preparation methods in terms of encapsulation efficiency, per cent release (at $T = 37°C$ and pH = 7.4) and mean size diameter of vesicles. As shown in Table 1, liposomal formulation, containing DPPC phospholipid, prepared with pH gradient method, forming small size diameters, showed extremely high encapsulation efficiency and made the drug release slower for both formulations as compared to other formulations.

A little longer hydrophobic part in the SPC than DPPC increases the repulsion of hydrophilic molecule of doxorubicin. Thereby, the vesicle size diameter makes larger and encapsulation efficiency decreases. Similar reports can be found indirectly from previous researches.[13]

The acyl chains were approximately equal in length for both phospholipids, but the acyl chain in SPC was unsaturated which made it more flexible and mobile than DPPC. This increased the drug leakage during preparation; as a result, the final encapsulation efficiency decreased and made the drug release fairly rapidly. Also the transition temperature of DPPC, unlike the SPC,[8,19] was higher than 37°C. So, the in vivo half-life of liposomes, synthesized with DPPC, was longer than that of SPC.

3.1.2 | Effect of phospholipid: cholesterol molar ratio

Various liposomal formulations were prepared with pH gradient methods and were compared to DPPC phospholipids in terms of encapsulation efficiency, mean size diameter, and percentage of release during 6, 24, and 48 hr. According to the results provided in Table 2, the liposome formula containing DPPC and cholesterol at a molar ratio of 1:1 (F2 and F8) showed highest drug encapsulation which decreased with increasing and decreasing the cholesterol content. Thus, the 1:1 molar ratio resulted in an optimum in encapsulation efficiency versus cholesterol content curve (Figure 1). Similar results were found previously.[20]

This behavior is attributed to the fact that the rigid chain in cholesterol structure makes the liposome more stable and

TABLE 1 Encapsulation efficiency and size of various phospholipid types and preparation methods

Formula	Preparation method	SPC:cholesterol (mole ratio)	DPPC:cholesterol (mole ratio)	EE%	Size (nm)	%Release (6 hr)	%Release (24 hr)	Release (48 hr) %
1	Thin film	0	1:0.67	17.25	132.5	55.78	61	68.04
2	pH gradient	0	1:0.67	86.51	121.7	49.30	53.27	55
3	Thin film	1:0.67	0	14.9	179.1	67	77	85.5
4	pH gradient	1:0.67	0	64.67	149.37	56.38	63.97	71.43

TABLE 2 Effect of phospholipids: cholesterol ratio and DSPE-mPEG (2000) on EE%, size, long-term and short-term release

Code.	DPPC:cholesterol	DSPE-mPEG (2000) (% mol)	EE%	Size (nm)	%Release (6 hr)	%Release (24 hr)	Release (48 hr) %
F1	1:1.5	0	75.385 ± 3	136	64	70.4	75
F2	1:1	0	92.70 ± 2	131.3	60.73	66.23	69.01
F3	1:0.67	0	86.51 ± 2	121.7	49.30	53.27	55
F4	1:0.43	0	77.84 ± 2	101.09	47.52	51.41	53
F5	1:0.25	0	68.82 ± 4	99.8	45.69	49.2	51.08
F6	1:0	0	57.6 ± 2	87.8	6.75	18.25	31
F7	1:1.5	3	82.36 ± 2	127.5	51.5	62.6	67
F8	1:1	3	95.32 ± 2	125.05	57.45	61.20	63
F9	1:0.67	3	88.87 ± 2	107.43	41.7	45.16	51.07
F10	1:0.43	3	82.80 ± 5	93.61	44.68	48.23	50.05
F11	1:0.25	3	73.16 ± 4	89	32.33	37.12	39
F12	1:0	3	62.3 ± 2	81.2	2.5	12.5	25

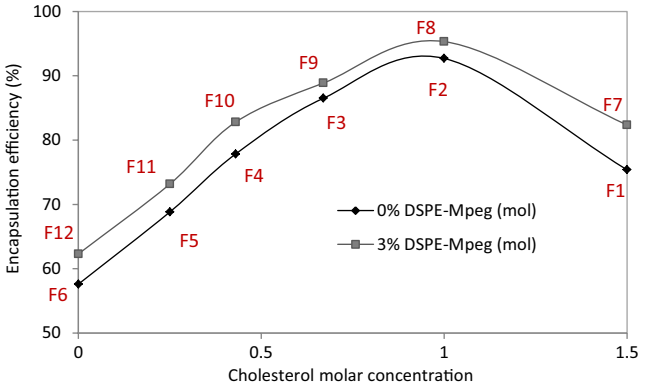

FIGURE 1 Effect of cholesterol variation content on encapsulation efficacy at constant DPPC molar content (comparison between F1 and F6 or F7–F12 formula)

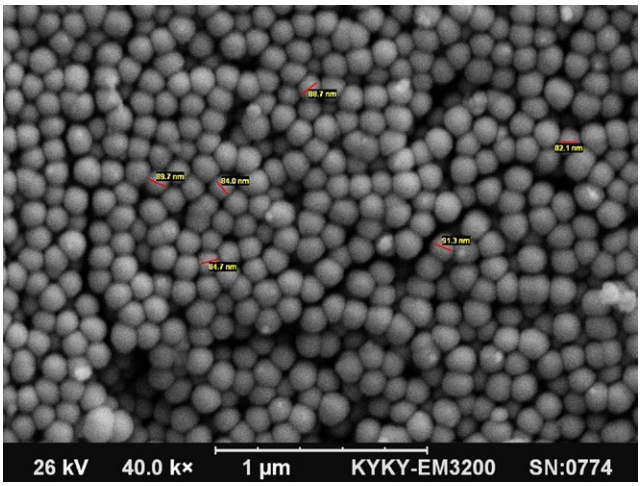

FIGURE 2 SEM micrograph of optimal formula (F10)

rigid and thus plays as an ameliorative to increase drug encapsulation efficiency, but at the same time increases the mobility and drug release especially in the short term, in contrast to the controlled drug release purpose. As can be seen from presented results, the mean diameter of liposomes increased with increasing the cholesterol content (F6→F1 or F12→F7, Table 2).

Cholesterol in low concentration (cholesterol molar concentration <1) facilitated the membrane permeation, led to high drug loading but increased drug leakage during preparation. This made the final drug encapsulation low. In high cholesterol concentrations (cholesterol molar concentration >1), acyl chain movement was limited. High amount of cholesterol also leads to more penetration into the inner layers of vesicles thus reducing the capacity of drug accumulation. This phenomenon decreased the encapsulation efficiency (Figure 1).[21,22] Also the drug release increased with increasing cholesterol content (F6→F1 or F12→F7, Table 2). Cholesterol content in liposomal formulation improves in vivo and in vitro stability of liposomes.[23,24]

3.1.3 | Effect of DSPE-mPEG (2000) in liposomal formulation

Table 2 shows the effect of DSPE-mPEG (2000) content on liposomal formulation. In general, according to these results, DSPE-mPEG (2000) content made the liposome smaller and some decrease in short-term drug release (compare F1→F7, F2→F8, F3→F9, F4→F10, F5→F11 and F6→F12). PEGylation improved in vivo stability of nanoparticles.[25] According to the results, the PEGylation increased the drug encapsulation (due to increasing aqueous space) and decreased mean size diameter and drug release and made the liposomal DOX more stable.

According to the results, the PEGylated liposomal formulation containing DPPC and cholesterol with 1:0.43 (F10)

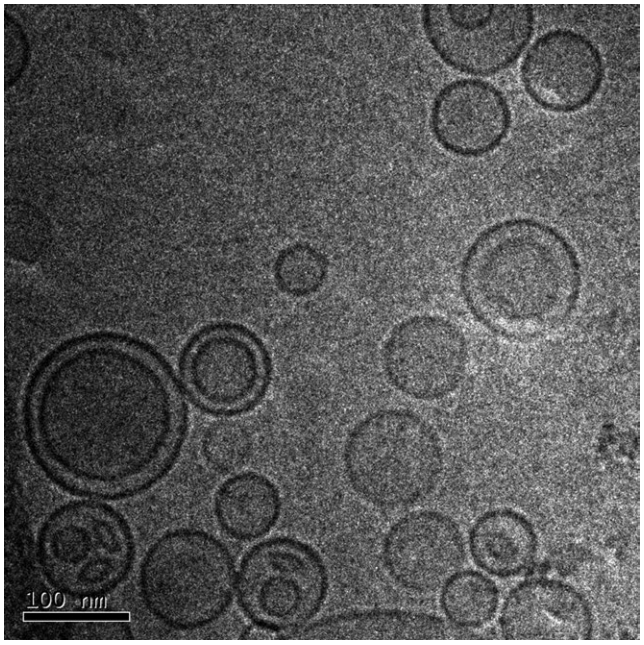

FIGURE 3 Cryo-TEM micrograph of optimal formula (F10)-bar = 100 nm

had approximately desired feature based on these three factors: small diameter, controlled drug release, and high encapsulation efficiency.

3.2 | Optimum formula

3.2.1 | Characterization of optimum formula

The mean size of the optimum formulation was 93.61 nm and 97.85 nm in number and volume mode, respectively, that is sufficiently small. The polydispersity index (PDI) was determined as the measurement index of homogeneity and

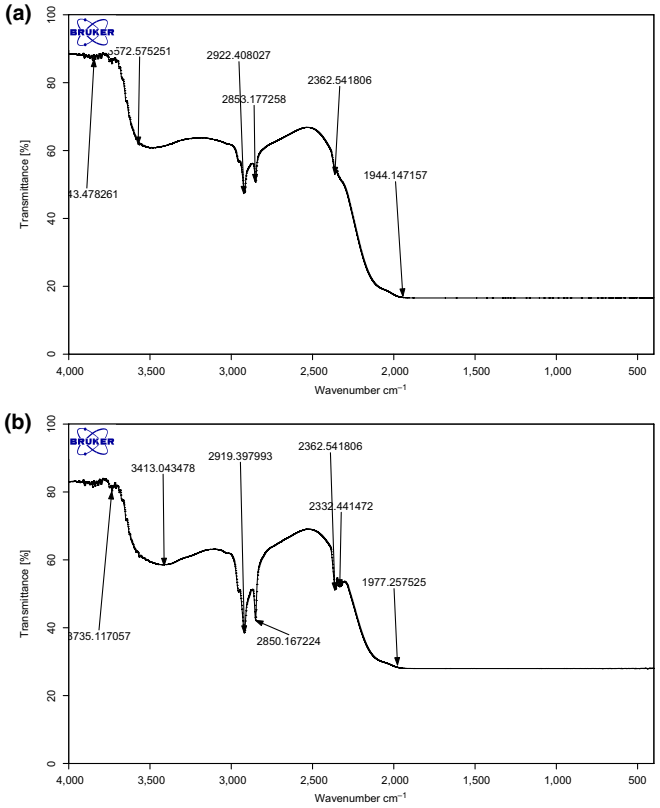

FIGURE 4 FTIR spectra of optimum formula (F10). (a) Before drug loading. (b) After drug loading

monodispersity. Its value for F10 formula was 0.141, which was lower than non-PEGylated form (F4, PDI=0.256). Small values of PDI demonstrated a homogeneous population in optimum formulation. Reducing PDI during PEGylation was due to steric hindrance, created by DSPE-mPEG (2000). Also the zeta potential of selected formula (F10) was −23.

According to SEM micrograph, the liposomal vesicles were found to be round, having smooth surface with no formation of aggregates as shown in Figure 2. As illustrated in this figure, the liposomal vesicle had well-identified rigid boundaries.

Cryogenic transmission electron micrographs of selected doxorubicin are shown in Figure 3. It was indicated that the particles were in a perfect spherical shape with large internal aqueous space and had a dispersed state. This figure also confirmed that the vesicle size of liposomes was approximately 93 nm.

3.3 | FTIR spectral evaluation

Figure 4a, b shows the FTIR studies of the optimal liposomal doxorubicin formula (F10). According to Figure 4a, in which the spectrum before DOX loading is shown, there were characteristic peaks of phospholipid, cholesterol and DSPE-mPEG at 3700 cm^{-1} (O-H stretching), 3400 cm^{-1} (N-H stretching), 2919 cm^{-1} (-CH3 asymmetric and symmetric stretching) and 2850 cm^{-1} (-CH2 asymmetric and symmetric stretching). These peaks were repeated in Figure 4b, which displays the FTIR spectrum after DOX loading.

In addition, in comparison with Figure 4a, b, the results clearly confirmed that there were no additional peaks and no chemical interactions between the drug loaded, and DPPC, cholesterol and DSPE-mPEG liposome. These results also confirm that the doxorubicin was stable during formulation.

3.4 | The thermosensitivity of the liposomal evolution

The thermosensitivity of the liposomal formulation was evaluated using differential scanning calorimetry for the

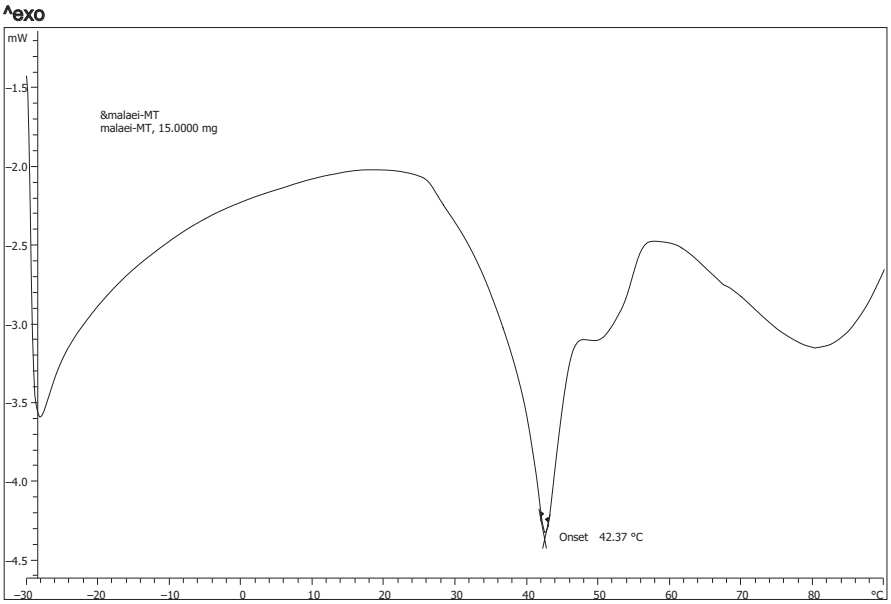

FIGURE 5 Differential scanning calorimetric scan (DSC) analysis of liposomes, composed of DPPC, cholesterol and DSPE-mPEG (2000)

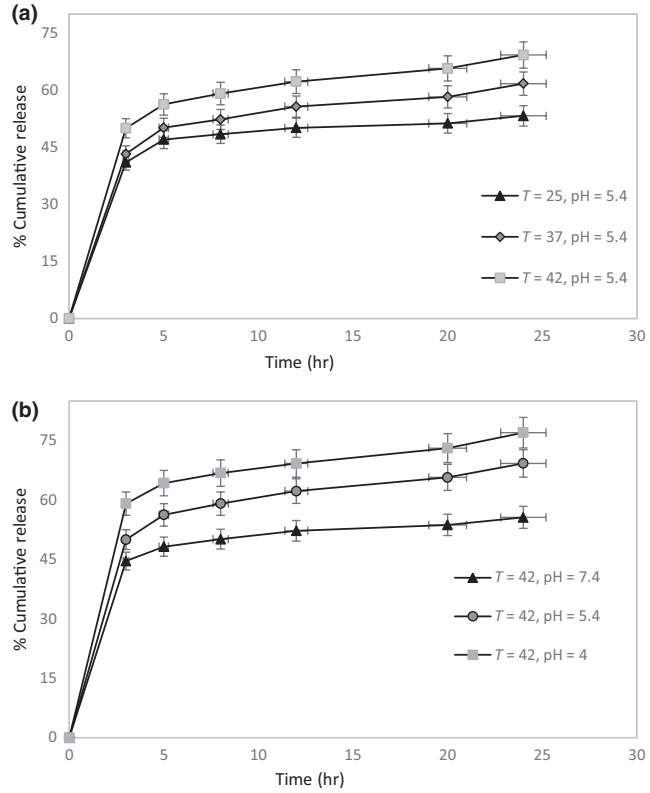

FIGURE 6 In vitro kinetic release of drug in various pH (a) and temperatures (b)

determination of phase transition temperature. As shown in Figure 5, a relatively sharp peak at 42.37°C was related to the transition temperature of phospholipid (DPPC) that showed the thermosensitive nature of the F10 formula (DPPC: cholesterol: DSPE-mPEG with 70:30:3 molar ratio). Peng et al. similarly demonstrated the thermosensitivity nature of prepared liposomal formulation by DSC analysis.[26]

3.5 | In vitro thermo- and pH-sensitive DOX release assay

The pH levels analyzed were chosen with care: the physiological pH of 7.4 is the condition which represents the level experienced in the blood stream; the pH 5.4 level is the value which the nanoparticles will encounter in the tumor area, while pH 4 is the pH level which is typical for lysosomes in which the liposomes will end up intracellularly.

Figure 6 showed the in vitro drug release of the selected formulation (F10) at various pH values (i) (4, 5.4, and 7.4) and temperatures (ii) (25, 37, 42°C). The kinetic analysis showed that the drug release follows two mechanisms, that is, drug pouring out from the liposome membrane and transferring inside the external buffer, controlled by diffusion (DOX concentration gradient between liposome and buffer) and convection mechanisms (slight shaking of external buffer), respectively.

Cancerous cells are faced with a lack of oxygen named hypoxia that led to pH drop inside cancer site. The pH and thermosensitivity nature of F10 reduced its activity in physiological condition, and it subsequently increased damage to malignant cells.

As can be seen, the rapid drug release took place at low pH and high temperature range, that is, the simulated lysosome (pH = 4.2) and cancer levels (pH = 5.4), while at 25°C and pH = 7.4, less drug release occurred. It can also be deduced that the new liposomal formulation could act as non-passive targeting for delivery to the endosomal compartments of the (cancerous) cells, while low drug release would occur at room temperature conditions (25°C). Thus, our results show that in particular at the lower pH levels, release is significantly higher, which ensures proper timing of release within the tumor and tumor cells, while avoiding high systemic exposure to DOX.

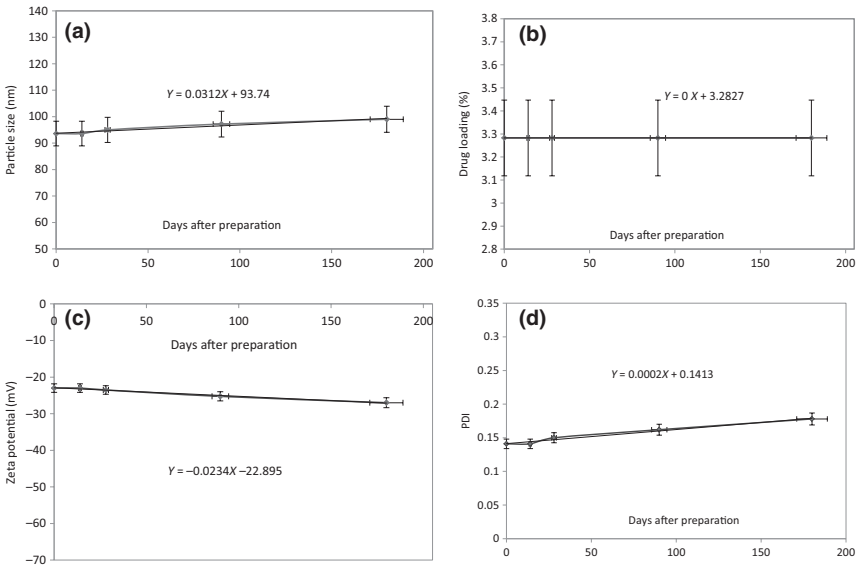

FIGURE 7 Stability study of liposomal doxorubicin (F10), stored at 4°C for 6 months. (a) Change of particle size. (b) Change of encapsulation efficiency. (c) Change of zeta potential. (d) Change of PDI. X axis are days after preparation

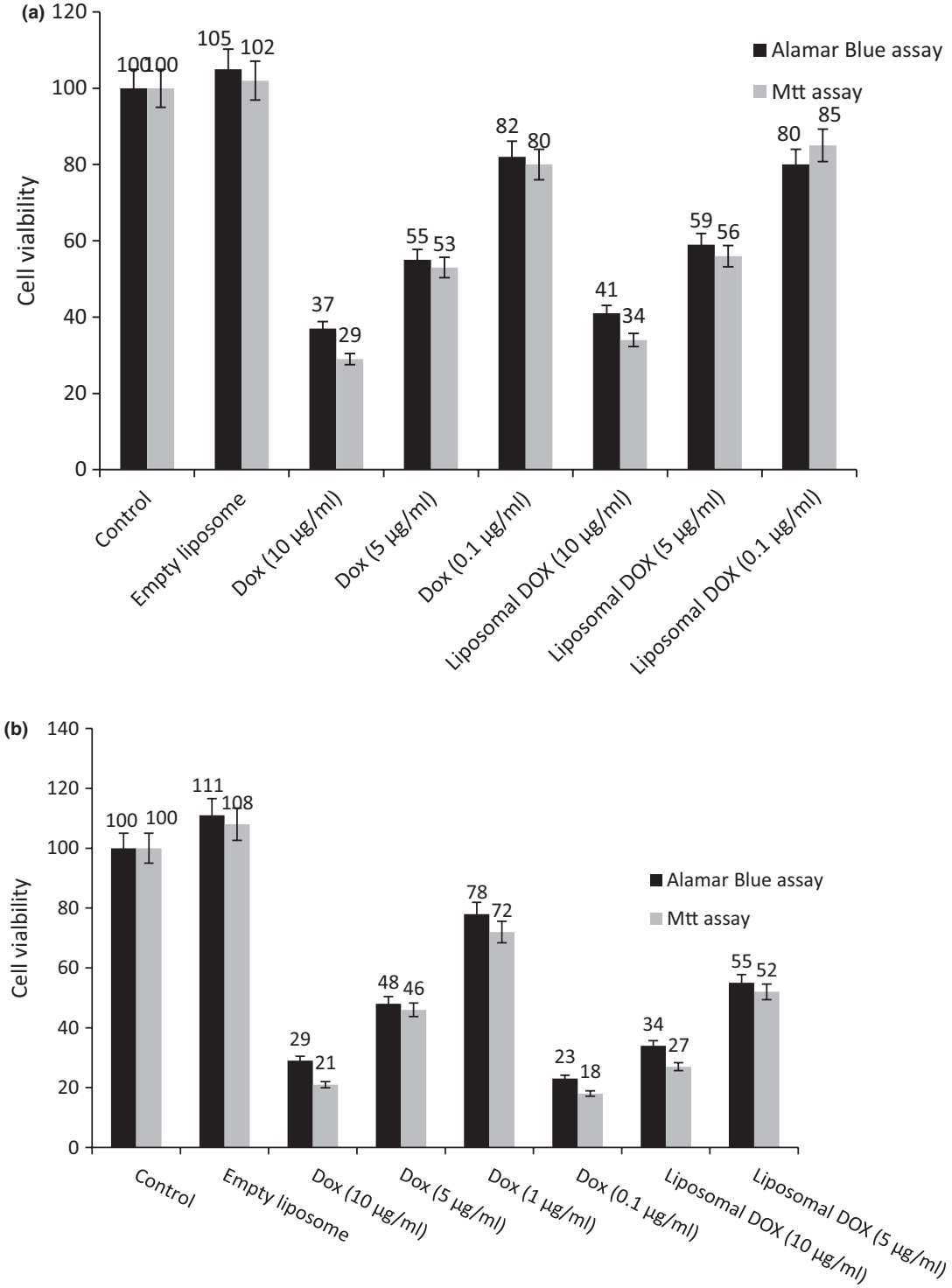

FIGURE 8 Comparison of MTT and Alamar Blue colorometric assays of MG-63 cells, (a) 24 hr cytotoxicity assay, (b) 72 hr cytotoxicity assay

3.6 | Physical stability

As indicated in Figure 7, after storage for 180 days, the mean vesicle size and encapsulation efficiency of optimized formulation (F10) was not significantly changed (less than 5.7% and 3.4%, respectively) from freshly prepared samples. The changes in PDI and zeta potential were approximately 26.2% (PDI still remains less than 0.3) and 17.4%, respectively. Based on these results, slopes of all curves were close to zero and intercepts of them were near to initial value of evaluated parameters and confirmed the stability of the F10 formula. This implies that the new liposome formulation F10 could minimize problems associated with liposome instability.

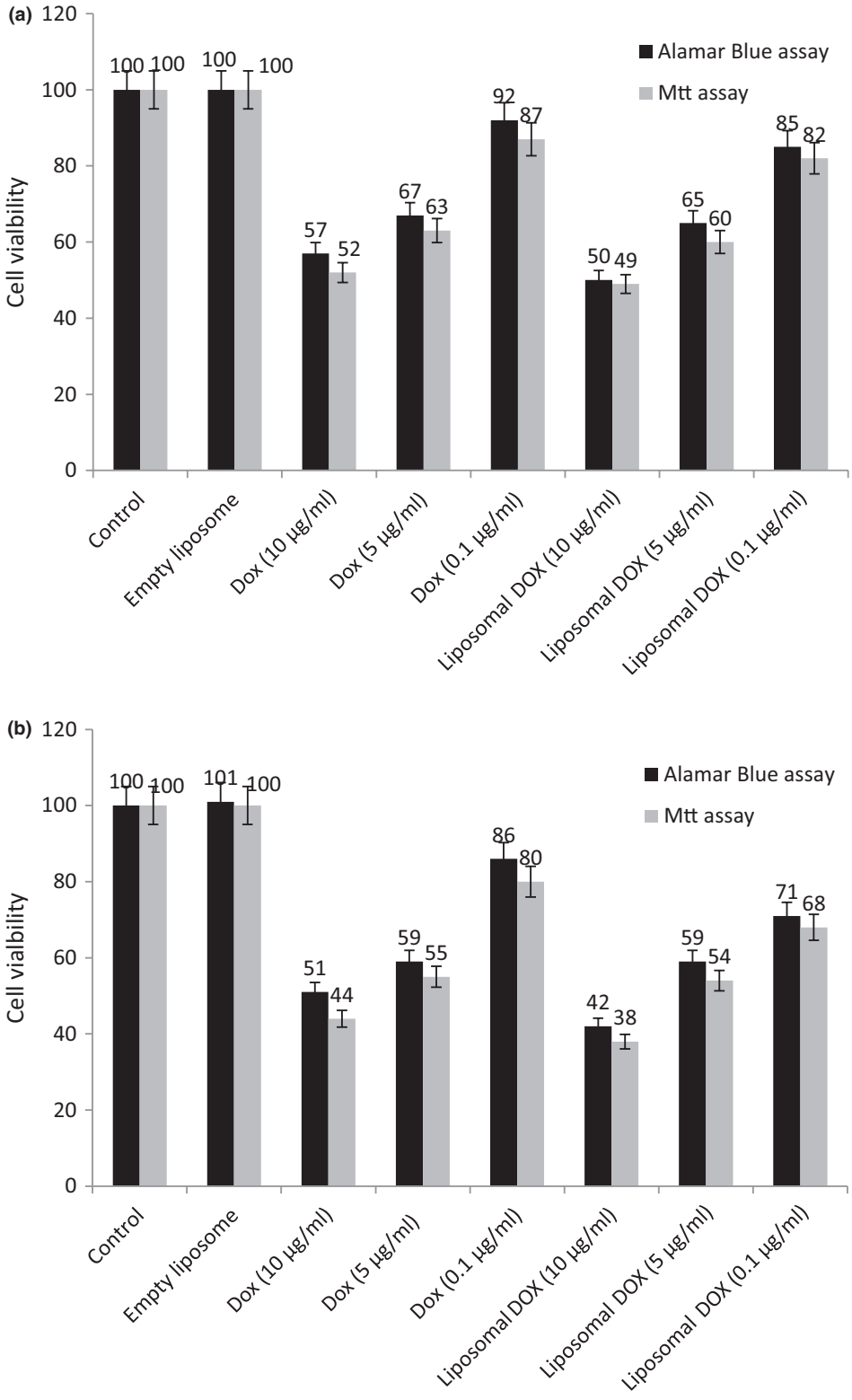

FIGURE 9 Comparison of MTT and Alamar Blue colorometric assays of primary bone cells, (a) 24 hr cytotoxicity assay, (b) 72 hr cytotoxicity assay

3.7 | Toxicity study

Figure 8a, b shows the cell viability in the presence of free DOX, blank liposome, and liposomal DOX with MTT and Alamar blue assay during 24 and 72 hr. The MTT and Alamar blue assay revealed that the proliferation of MG-63 cell line was inhibited with liposomal DOX and free DOX. Results (Figure 8a, b) show that blank liposome had no toxicity and could improve

cell proliferation. Generally, as indicated in Figure 8a, during 24-hr period, the liposomal formulations indicated lower growth inhibition than free DOX. This could be simply explained by the slow release rate of free DOX-loaded liposome.

Also after 72-hr incubation, it was found that free DOX and liposomal DOX IC_{50} were approximately 5 and 0.1 μg/ml with both MTT and Alamar blue assays. Compared to free DOX, drug encapsulation in liposome enhanced the cytotoxicity (IC_{50}, decreased cell viability) of doxorubicin by approximately 1.33 (Alamar blue) and 1.38 (MTT)-folds. NB: As the more dilute samples had not any toxic effect and the more concentrated samples showed a 100% tumor cell kill efficiency, we showed only the relevant dosages necessary to estimate the IC50 values.

In comparison between MTT and Alamar blue colorimetric assays, the presented data show that the obtained IC_{50} in MTT assay in all time intervals and the concentrations were lower than Alamar blue assay. It can be concluded that MTT assay is more sensitive than Alamar blue assay but that there is good correlation between the results of two methods. These results confirmed previously reported researches.[27,28] MTT assay is fast, precise, and easy in determining the sensitivity and behavior of anticancer drugs on cancer cell lines.[29,30]

Because of the toxic nature of DOX and the concerns about true sensitivity and targeting ability of the F10 formulation, the cytotoxicity of the current formulation was also checked on primary bone cells and results represented in Figure 9. Results showed reduced cytotoxicity to healthy cells than just malignant cells.

4 | CONCLUSIONS

Our successful findings confirmed and extended the former evidence for the development of liposomal doxorubicin formulation. We reported a new formulation for stealth, thermo- and pH-sensitive liposomal doxorubicin to reduce drug dosage for cancer treatment, with enhanced therapeutic index and improved cytotoxicity effect on MG-63 osteosarcoma cell line. There was also no chemical interaction between drug and the carriers. Cancer treatment demands targeted, prolonged, and controlled release of anticancer drugs, which can be achieved through our new formulation. Results of DSC analysis and drug release profile confirm that our formulation is thermo and pH sensitive. The results of current study may encourage researchers to manufacture stable liposomal doxorubicin formulations produced with a novel method in an economically feasible manner.

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CONFLICT OF INTEREST

There is no conflict of interest.

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