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ORIGINAL RESEARCH

The role of size in PEGylated liposomal doxorubicin biodistribution and anti-tumour activity

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Abstract

The size of nanoliposome-encapsulated drugs significantly affects their therapeutic efficacy, biodistribution, targeting ability, and toxicity profile for the cancer treatment. In the present study, the biodistribution and anti-tumoral activity of PEGylated liposomal Doxorubicin (PLD) formulations with different sizes were investigated. First, 100, 200, and 400 nm PLDs were prepared by remote loading procedure and characterised for their size, zeta potential, encapsulation efficacy, and release properties. Then, in vitro cellular uptake and cytotoxicity were studied by flow cytometry and MTT assay, and compared with commercially available PLD Caelyx®. In vivo studies were applied on BALB/c mice bearing C26 colon carcinoma. The cytotoxicity and cellular uptake tests did not demonstrate any statistically significant differences between PLDs. The biodistribution results showed that Caelyx® and 100 nm liposomal formulations had the most doxorubicin (Dox) accumulation in the tumour tissue and, as a result, considerably suppressed tumour growth compared with 200 and 400 nm PLDs. In contrast, larger nanoparticles (200 and 400 nm formulations) had more accumulation in the liver and spleen. This study revealed that 90 nm Caelyx® biodistribution profile led to the stronger anti-tumour activity of the drug and hence significant survival extension, and showed the importance of vesicle size in the targeting of nanoparticles to the tumour microenvironment for the treatment of cancer.

KEYWORDS

Biodistribution, Cancer, EPR effect, Nanoparticle size, PEGylated liposomal doxorubicin, Therapeutic efficacy

1 | INTRODUCTION

Cancer is the leading cause of major health problems and mortality throughout the world. It is a complex disease caused by the accumulation of genetic mutations and perturbation of cell cycle leading to the tumour proliferation and development of impaired immunity and its survival [1, 2]. There are numerous efforts aiming to develop effective therapeutics targeting different kinds of cancer. Chemotherapy, along with surgery and radiation, is often the first line of defence in the treatment of cancer. It works by gradually demolishing the cancer cells at the original tumour site and preventing them from spreading or decelerating their growth. However, even as a major treatment option among different approaches, it has several limitations such as cardiotoxicity, fatigue, hair loss, infections, nausea, vomiting, and so forth that need further investigations and improvements [3, 4]. Nanotechnology offers appropriate solutions to many limitations and problems in cancer treatment.

Nanoparticles can accumulate within tumour tissues through the enhanced permeation and retention (EPR) process, also known as passive targeting. Tumour's leaky vasculature and deficient lymphatic drainage enhance nanoparticle penetration and accumulation in the tumour microenvironment [5]. Studies have demonstrated that the size of these nanoparticles is crucial for a potent EPR effect. Different tissues are reported to have distinct cut-offs for an optimised nano-drug delivery system that perfectly extravasates the tumour environment [6–8].

Recent advances in nanotechnology have shown that nanoparticles such as liposomes can improve clinical care and treatments [9]. Liposomes are one of the most-studied nano-based drug delivery systems (NDDSs) that are constructed from one or more phospholipid bilayers that contain an aqueous core. Therefore, liposomes are able to be loaded with hydrophobic and/or hydrophilic molecules and deliver them to the action site. The phospholipids can originate from natural sources, which are biologically and immunologically inert, and show lower inherent toxicity [10–12]. Due to their nature and physicochemical properties, liposomes are considered as safe, biocompatible, and biodegradable nanostructures that are amenable to broad pharmaceutical applications [13–15]. Different liposomal drug formulations provide the possibility of enhancing efficacy and decreasing severe systemic side effects of anticancer drugs [16, 17]. Furthermore, it has been proved that administered liposomal drug delivery systems in comparison with free drugs have drastically improved the anticancer medication pharmacokinetics [18]. Many studies have shown that among several Food and Drug Administration (FDA) approved NDDSs, Caelyx®, PEGylated liposomal doxorubicin (PLD), reaches higher drug concentrations in the tumour mass and better therapeutic index than conventional (non-PEGylated) liposomal Dox or free Dox [19, 20].

Caelyx® is a long-circulating PLD, containing surface-grafted segments of the hydrophilic polymer methoxy polyethylene glycol (mPEG), with an average particle size of approximately 90 nm [21, 22]. The impact of size in pharmacokinetics, tissue extravasation, uptake, and clearance rate from the injection site has been documented before. This study confirms the liposomal particle size plays a vital role in cancer treatment and biodistribution [23, 24].

Although several studies have been published regarding the liposomal size impacts on the therapeutic efficacy, biodistribution, release and toxicity profiles of PEGylated liposomal Dox, none has provided a comprehensive and well-organised overview. As a result, we conducted this study to gain an integrated understanding of the significance of vesicle size in Dox distribution to tumour and tissue sites. Therefore, in this study, the PLDs with different sizes (100, 200, and 400 nm) were developed, and their in vitro and in vivo characteristics such as cell uptake and toxicity, biodistribution, and therapeutic efficacy were compared relative to Caelyx® and free Dox. Flowcytometry was used to investigate the formulations' cellular uptake. Additionally, MTT assay was performed to evaluate cytotoxicity. In vivo studies were performed on BALB/c mice bearing C26 colon carcinoma. After the injection of a single dose free Dox, Caelyx®, and different sizes of PLDs, the biodistribution and therapeutic efficacies were determined.

2 | METHODS AND MATERIALS

2.1 | Materials

Caelyx® was acquired from Behestan Darou (Tehran, Iran). Hydrogenated soy phosphatidylcholine (HSPC) and Methoxypolyethylene glycol-1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine (mPEG2000-DSPE) were purchased from Avanti polar lipids (Alabaster, AL). Cholesterol was from Sigma-Aldrich (St Louis, MO). Isopropanol was obtained from Merck (Darmstadt, Germany), and it was acidified by the addition of 7.5 ml HCl 1M and 2.5 ml water to 90 ml isopropanol. Doxorubicin HCl was purchased from Sigma-Aldrich. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was bought from Promega (Madison, WI). C26 mouse colon carcinoma cells were acquired from Cell Lines Service (Eppelheim, Germany), and they were cultured in Stands for Roswell Park Memorial Institute Medium (RPMI) 1640 medium (Sigma-Aldrich). The media was supplemented with inactivated Foetal Calf Serum (FCS) (Sigma-Aldrich) and penicillin/streptomycin (Merck). All other solvents and reagents were provided and applied as the chemical grade.

2.2 | Different PEGylated liposomal doxorubicin sizes preparation

To prepare the thin lipid film, stock lipid solutions in chloroform including HSPC, mPEG2000-DSPE, and cholesterol at a molar ratio of 56.2/38.3/5, as is used in Caelyx®, were mixed in a round bottom flask. The solvent was removed by a rotary evaporator under reduced pressure to obtain a thin film on the flask wall. The solvent residuals were removed by connection to a vacuum pump through a freeze-dryer. Next, the lipid film was hydrated in 250 mM ammonium sulphate buffer at 65°C, vortexed for 30 min, and sonicated in a water bath (68°C) for 15 min. Before extrusion, the milky white suspension was frozen and thawed for three cycles. A single freeze-thaw cycle consisted of freezing at liquid nitrogen temperature (−196°C) and thawing in a water bath at 65°C. To prepare liposomes with pre-specified sizes (100 nm, 200 nm, and 400 nm), the suspension was extruded 11 times through different size polycarbonate membranes using the LIPEX™ extruder. These processes were performed at 65°C. The prepared liposome formulation was then dialysed three times using 12–14 KDa molecular weight cut-off (MWCO) to exchange ammonium sulphate with histidine buffer (pH 6.5). As the final step, the phospholipid contents of liposomal formulations were determined by Bartlett phosphate assay [25]. For remote loading of Dox into prepared liposomes, the appropriate Dox concentration was added to the liposomes and were co-incubated at 65°C for one hour with gentle shaking, then purified using dialysis (12–14 KDa MWCO) against dextrose-histidine buffer. Moreover, Caelyx® with the size of 90 nm was used as the presentative of the smallest nanoliposomal formulation.

2.3 | Liposomal characterisation

Size distribution, zeta potential, and PDI of the prepared liposomal formulations were calculated by dynamic light scattering (Nano-ZS; Malvern, UK). Transmission electron microscopy (TEM) was used to evaluate the morphological properties of the

prepared liposomes [26]. In brief, the samples were prepared for TEM by putting a drop of each diluted PLD onto a carbon-coated copper grid, followed by removing excess samples, and placing a drop of uranyl acetate on the grid for negative staining. Finally, the samples could be seen using LEO 912 TEM at an acceleration voltage of 80 kV (Zeiss, Jena, Germany). Additionally, each formulation's encapsulation efficiency (EE%) was determined by measuring the Dox concentration with spectrofluorimetry (Shimadzu RF5000U, Japan) at excitation of 490 nm and emission of 585 nm, before and after removing excess Dox from liposomal formulations with dialysis:

$$\text{EE\%} = (\text{amounts of Dox after dialysis} / \text{amounts of Dox added}) \times 100$$

2.4 | Drug release studies

The release experiment was conducted according to two protocols. Firstly, it was done in 50% human plasma as the release media, which had 5% dextrose (1:1 v/v). The drug-loaded liposomes were added to this media (1:9 v/v) and incubated at 37°C. After that, 1 ml of each sample was collected from the release media in predetermined time points (1, 2, 4, 6, and 24 h), mixed with Dowex® resin, and rotated for 15 min. Finally, the supernatants were assessed for released Dox from the liposomes with spectrofluorimetry (Shimadzu RF5000U, Japan) at excitation of 490 nm and emission of 585 nm [27].

Secondly, the release study was done by the dialysis method in three different media with a pH of 5.5 (dextrose succinate), 6.5 (dextrose histidine), and 7.4 (PBS). To this end, each formulation was put into the dialysis bag (cut off 12–14 kDa), and immersed in the aforementioned buffers, and then incubated at 37°C overnight. Samples from the release media were collected at defined time points (1, 2, 4, 6, and 24 h), and the dialysis buffer was refreshed. Eventually, the amount of released Dox was analysed with spectrofluorimetry (Shimadzu RF5000U, Japan) at excitation of 490 nm and emission of 585 nm [28].

2.5 | MTT cytotoxicity test

C26 cells were seeded at a 5000 cells/well density in 96-well plates. After an overnight incubation, the cells were treated with serial dilution of Caelyx®, free Dox, and the PEGylated liposomal formulations in triplicate and incubated at 37°C for 48 h. After finishing the incubation time, the media was removed, the cells were washed with cold PBS, and the mixture of MTT and FCS-free medium was added to each well, and then incubated for 4 h at 37°C. Finally, the medium was substituted with DMSO, and the plates were shaken for 30 min. Eventually, the absorbance of each well was determined at 570 nm using a Multiskan Plus plate reader (lab systems). The inhibition rates were calculated as follows:

$$\text{Rate of inhibition} = 1 - \left(A_{\text{sample}} - A_{\text{blank}}\right)/\left(A_{\text{control}} - A_{\text{blank}}\right)$$

Each formulation's IC50 value was calculated using CalcuSyn version 2.0.

2.6 | Flow cytometry assay

Flow cytometry analysis was applied to determine the specific cellular uptake of the prepared PEGylated nanoliposomal formulations. Concisely, C26 cells were seeded in 24-well plates at a density of 2.5×10^5 cells per well in a complete medium containing 10% FCS, overnight. The cells were treated with the drug (Dox, Caelyx®, and the formulations) at 37°C for 3 h. Afterwards, the cells were washed three times with cold PBS, detached by 0.5% EDTA-trypsin (Gibco, UK), and centrifuged at 1500 rpm for 5 min. Finally, the cell-pellets were washed three times with PBS containing 1% FCS, and the geometric mean fluorescence intensity of Dox in cells was analysed by flow cytometry (FACSCalibur, BD) in the FL2 channel.

2.7 | In vivo studies

2.7.1 | Ethical statements

BALB/c mice were purchased from the Pasteur Institute (Tehran, Iran). All animal experiments complied with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines, were approved by the Institutional Ethical Committee and Research Advisory Committee of Mashhad University of Medical Sciences, and were performed in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments. Microenvironments (housing, cage, enrichment, and cleaning) and macroenvironments (temperature, ventilation, and humidity) were provided based on guidelines.

2.7.2 | Chemotherapy study

C26 colon carcinoma cells were subcutaneously injected into female BALB/c mice (4–6 weeks old) in their right flanks (3×10^5 cells/mouse). Ten days post-inoculation, mice were randomly allocated to different treatments groups ($n = 5$) and received a single dose of Caelyx®, 100, 200, and 400 nm PLDs, free Dox (10 mg/kg, iv single dose), and equivalent volumes of saline as the control group, intravenously. Weight and tumour size were observed and checked over 60 days. Precisely, three orthogonal diameters were measured with digital callipers, and the tumour volume was measured as (length × width × height) × 0.52. Mice were sacrificed when they met the exclusion criteria, including bodyweight loss >20% of their initial weight and tumour size of >1000 mm^3. Moreover, the time to reach the endpoint (TTE) for each mouse was calculated based on the line equation attained by tumour growth curve exponential regression [29]. The median survival time (MST) was calculated using Kaplan-Meier analysis, and the tumour growth delay

percentage (%TGD) was measured according to the below formulation [30]:

$$\%TGD = [(\text{mean TTE of treatment group} - \text{mean TTE of control group}) / \text{mean TTE of control group}] \times 100$$

Moreover, the increased life span (%ILS) was obtained from the following equation [31]:

$$\%ILS = [(\text{MST of treatment group}/ \text{MST of control group}) \times 100] - 100$$

2.7.3 | Biodistribution study

Fourteen days after tumour inoculation, the mice were administered with a single dose of Caelyx®, PLDs (100, 200, and 400 nm), free Dox, and equivalent volumes of saline ($n = 3$). The blood samples were obtained at 1 and 3 h after injection via retro-orbital bleeding. After 24 and 48 h, the mice were sacrificed, and the blood samples were obtained by cardiac puncture. Different organs, including tumour, heart, kidney, liver, spleen, and lungs were separated, weighed, and placed in 2 ml polypropylene micro vials (Biospec, OK) containing 1 ml acidified isopropanol and zirconia beads and homogenised with Mini-Beadbeater (Biospec, OK). The samples were then centrifuged, and the supernatant was determined for Dox concentration, spectrofluorimetrically (Ex: 470 nm, Em: 590 nm). The calibration curve was provided using serial dilutions of Dox in the tissue [27].

2.7.4 | Pharmacokinetic study

To perform pharmacokinetic analysis, plasma samples were collected from each mouse after 1-, 3-, 24-, and 48-h post-injection and analysed for Dox concentration using spectrofluorimetry (Ex: 470 nm, Em: 590 nm). The non-compartmental model was chosen to perform the pharmacokinetic analysis. For each formulation, some parameters, namely the area under the plasma concentration versus time curve (AUC), the area under the first moment curve (AUMC), mean residence time (MRT), total clearance (Cl), the volume of distribution (V) and the elimination half-life ($t_{1/2}$) were measured. The results were represented as the mean ± standard deviation.

2.8 | Statistical analysis

Statistical analysis was conducted with GraphPad Prism, version 5 (San Diego, CA). The other comparisons were estimated by one-way analysis of variance. The survival data were also examined by the log-rank test. The survival results were estimated by Kaplan-Meier analysis.

3 | RESULTS

3.1 | Liposomal characterisation

Size, polydispersity index (PDI), and zeta potential of various liposomal formulations were assessed. As is shown in Table 1, the z-average of 100, 200, and 400 nm PEGylated liposomal formulations was 124, 235, and 446 nm, respectively, with values of PDI ≤0.2, and zeta potential of −17 mV. Moreover, the images taken by TEM (Figure 1) confirmed that the prepared PLD formulations were almost uniform and spherical in shape with the sizes of around 90, 100, 200 and 400 nm, which were compatible with the results of Dynamic Light Scattering (DLS) shown in Table 1. The results also demonstrated achievable remote loading and proper encapsulation of Dox into the different formulations. Dox encapsulation efficiencies of 100 nm, 200 nm, and 400 nm liposomal formulations were 99%, 98% and 90%, respectively. More specifically, the highest Dox content was 1.9 mg/ml for 100 nm, and the lowest was found at 1.6 mg/ml for 400 nm. These findings were conformable to the results of other experiments, which illustrated that remote loading is the most appropriate method to achieve the desired high concentrations of drug per nanoliposome [32].

3.2 | Release studies

In vitro drug release is often established using a test solution that reflects different physiological conditions, which can assess the quality, efficacy, and stability of the products. Figure 2 illustrates the formulations' in vitro drug release

TABLE 1 Physicochemical properties of different PEGylated liposomal Dox formulations (Data represented as mean ± standard deviation of 3 independent preparations)

Formulations	Z-average (nm)	Size by number (nm)	Size by volume (nm)	Size by intensity (nm)	PDI[a]	Zeta potential (mV)	Dox (mg/ml)	EE[b] (%)
100 nm	124.80 ± 0.38	82.20 ± 0.21	117.50 ± 0.18	140.70 ± 0.36	0.166 ± 0.003	−17.00 ± 6.29	1.90	99.00 ± 0.15
200 nm	235.50 ± 0.31	148.00 ± 1.05	206.50 ± 0.65	208.80 ± 0.59	0.103 ± 0.002	−17.80 ± 7.17	1.70	98.00 ± 0.72
400 nm	446.00 ± 1.07	176.00 ± 3.49	320.60 ± 4.30	295.60 ± 2.07	0.206 ± 0.003	−17.30 ± 5.37	1.60	87.00 ± 0.94
Caelyx®	90.20 ± 0.73	69.92 ± 0.31	83.23 ± 0.62	96.94 ± 1.12	0.064 ± 0.005	−17.20 ± 0.37	2.00	99.80 ± 0.60

[a]Stands for polydispersity index.

[b]Stands for encapsulation efficiency calculated according to Equation 1.

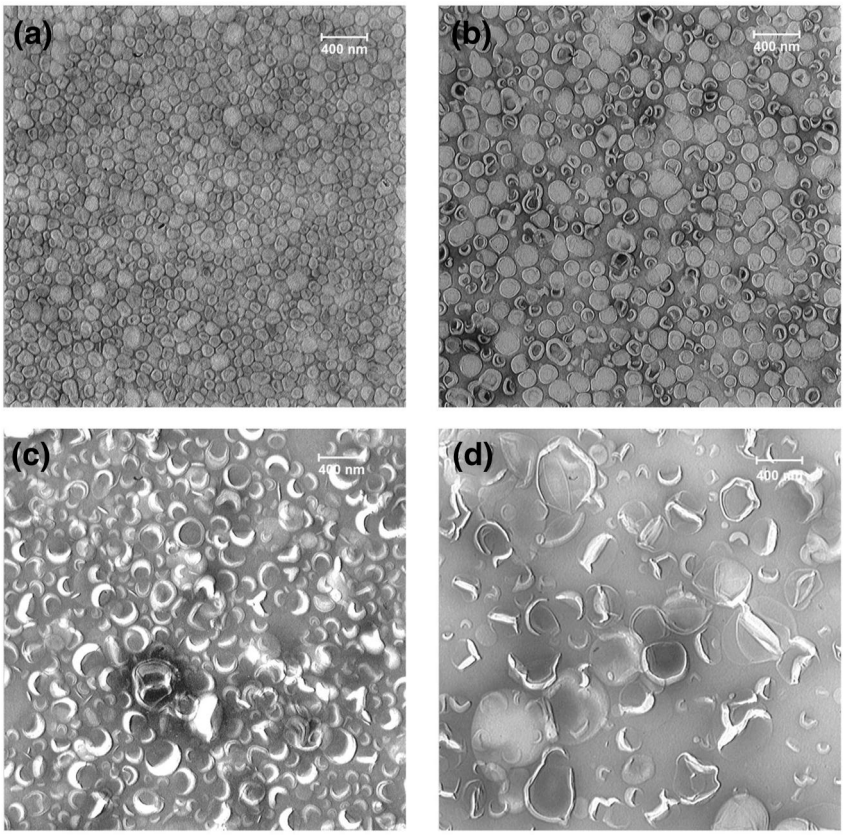

FIGURE 1 Transmission electron microscopy (TEM) images of PEGylated nanoliposomal formulations. (a) 100 nm, (b) 200 nm, (c) 400 nm, and (d) Caelyx®

profiles in 50% fresh human plasma for 24 h. As is shown, there was not any considerable difference between the release of Caelyx® and other liposomal formulations (less than 10%). In the first 5 h, all formulations' release profiles followed upward trends to 6%. The release profile of these figures did not change significantly over the next hours.

In order to simulate the physiological, tumoral, and endosomal conditions, the release profiles were evaluated at pHs of 7.4, 6.5, and 5.5, respectively. Interestingly, the PEGylated liposomal formulations' Dox release was low and less than 2% at different pHs.

3.3 | Cytotoxicity test

The formulations' cytotoxicity was tested on the C26 cells using the MTT assay. As detailed in Table 2, the Caelyx® toxicity was lower than that of Dox in vitro and in agreement with the results of previous reports [33]. Moreover, the results showed no significant differences in toxicity between Caelyx® and other PEGylated liposomal formulations against the C26 cell line after 48 h of incubation. The presence of PEG may explain the similar toxicity profile in all formulations.

3.4 | Cellular uptake

To determine cellular uptake by C26 cell line, the flow cytometry experiment was used for each formulation. As is shown in Figure 3, the PEGylated nanoliposomal formulations (including 100, 200, and 400 nm) did not demonstrate any significant difference in cellular uptake on the C26 cell line as compared to Caelyx®. However, the conventional form of Dox had the highest cell uptake in comparison with the other formulations.

3.5 | In vivo studies

3.5.1 | Chemotherapy study

The therapeutic efficacy was evaluated compared to the Caelyx® in murine C26 colon carcinoma tumour model. In order to achieve this goal, bodyweight, tumour volume, and survival were monitored over time. The data showed that there was not any significant body weight reduction over time, indicating that the drugs did not create extra toxicity (Figure 4).

Moreover, as depicted in Figure 4, treatment with 100 nm formulation in the C26 tumour model decreased tumour growth rate more effectively, which was approximately similar to the Caelyx® tumour volume profile. The survival results that were represented in a Kaplan-Meier plot were used to investigate survival rates. In the C26 tumour model, Caelyx®, 100, 200, and 400 nm formulations remarkably expanded mouse survival compared to PBS. The overall survivals were also significantly enhanced in Caelyx® treated mice in comparison with those who received the other formulations.

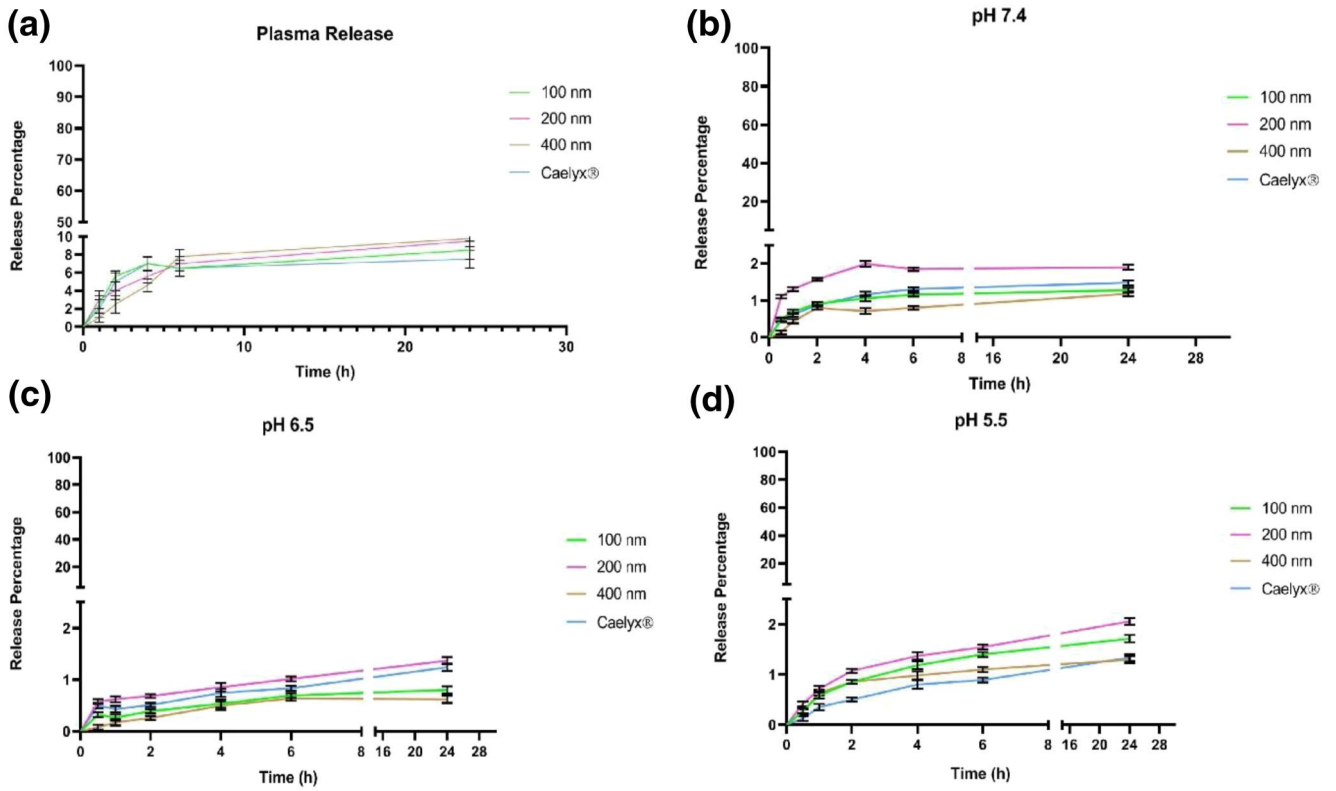

FIGURE 2 The release profile of different sizes of PEGylated nanoliposomal formulations in (a) human plasma, and at pHs of (b) 7.4, (c) 6.5 and (d) 5.5. The error bars were obtained from triplicate samples

TABLE 2 In vitro cytotoxicity effect (IC50) of different PEGylated nanoliposomal formulations, Caelyx® and free Dox against C26 Cells after 48h. Data represented as μ molar ± standard deviation ($n = 3$)

Treatment	Dox HCl	Caelyx®	100 nm	200 nm	400 nm
C26 (μg/ml)	0.16 ± 0.02	11.21 ± 1.10	8.60 ± 0.70	8.00 ± 1.00	9.20 ± 0.90

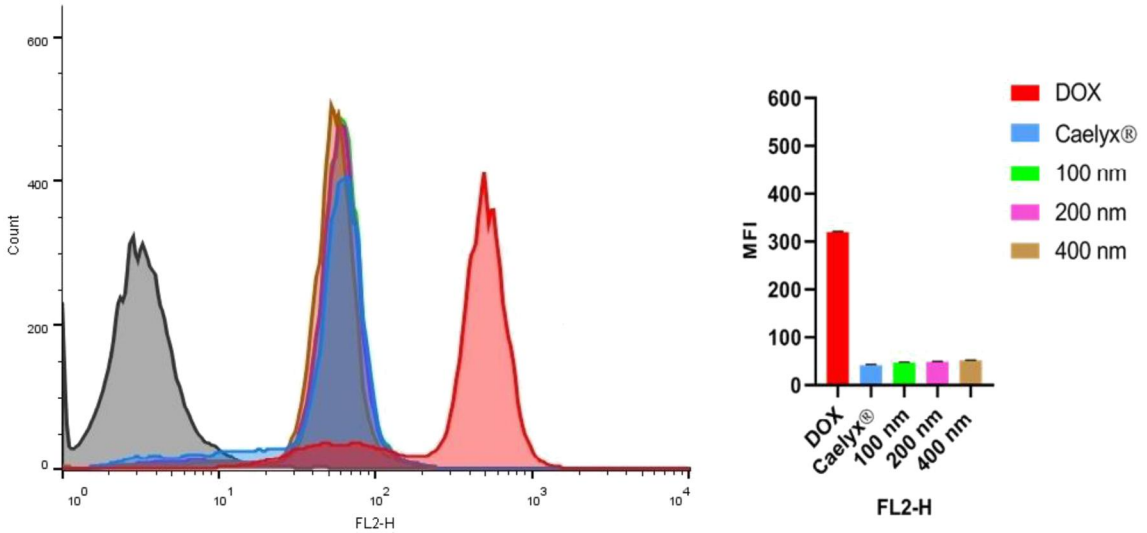

FIGURE 3 In vitro cellular uptake of different PEGylated nanoliposomal formulations, Caelyx® and free Dox by C26 cells at 37°C. Results expressed as geometric mean fluorescence intensity (MFI) ($n = 3$, mean ± SEM)

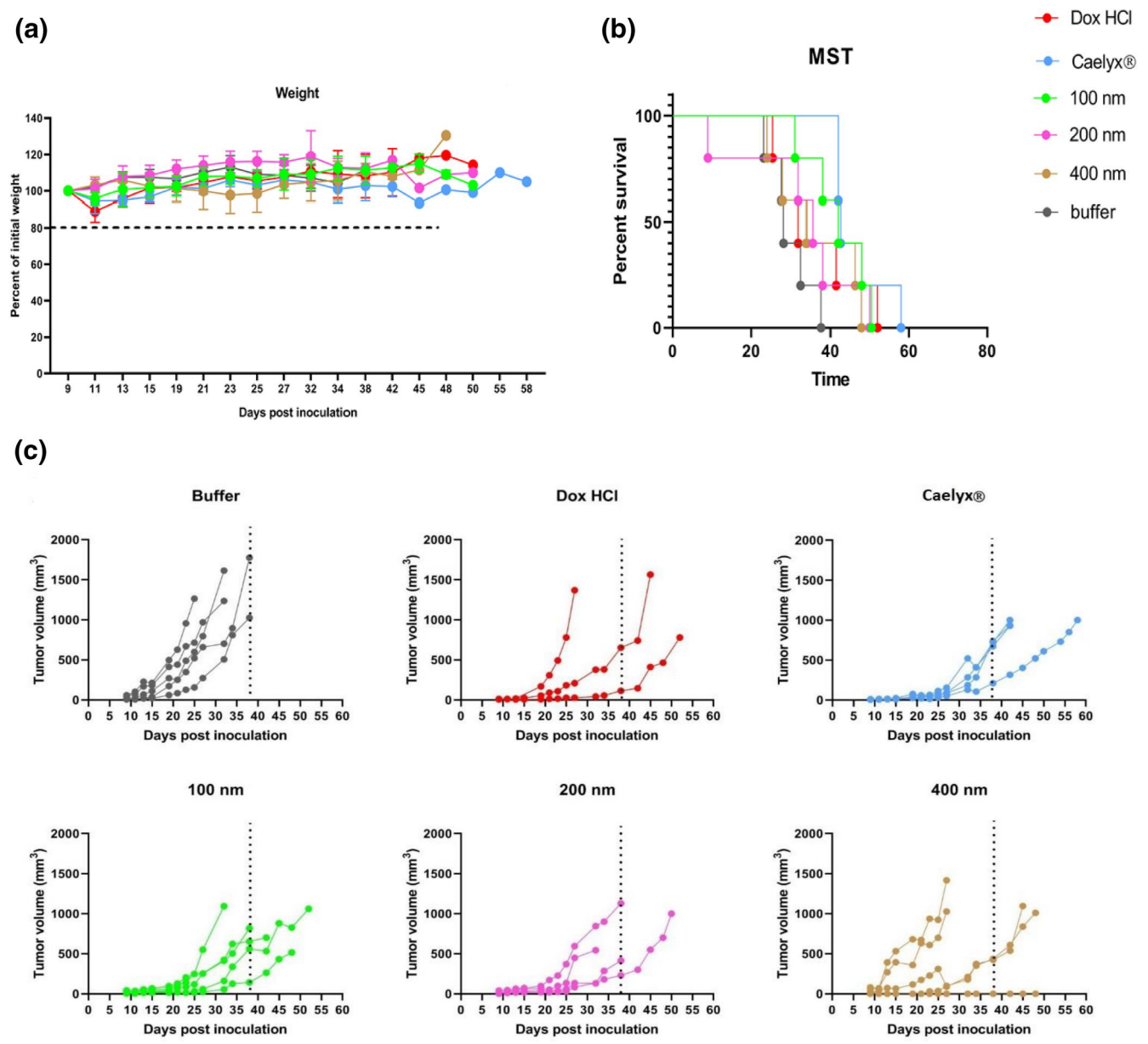

FIGURE 4 Therapeutic efficacy of different PEGylated nanoliposomal formulations, Caelyx® and free Dox in BALB/C mice bearing C26 tumour after iv administration of a single dose of 10 mg/kg on day 14 after tumour inoculation. (a) percent of changes in animal body weight, (b) survival curve, and (c) average tumour volume

A number of prominent indicators related to the therapeutic efficacy are shown in Table 3, including MST, TTE, % TGD, and %ILS. As can be seen in Table 3, the MST of Caelyx® was 48 days. The other formulations' (100 nm, 200 nm, and 400 nm) MST was significantly lower than Caelyx® at the same dose. The %TGD of Caelyx® was 92% and was decreased to 61%, 50%, and 28% for 100 nm, 200 nm, and 400 nm, respectively. Furthermore, 100, 200, and 400 nm treatments, and also Caelyx® increased 48.5%, 34.3%, 20.2%, and 69.6% the survival time compared to the PBS group, which means 13.7 days (100 nm), 9.7 days (200 nm), 5.7 days (400 nm) and 19.7 days (Caelyx®) expanding in the lifetime of mice receiving a single dose of liposomal formulations (10 mg/kg) as opposed to the control group. Overall, the 90 nm formulation (Caelyx®) was more efficacious at a 10 mg/kg single dose in mice bearing the C26 tumour model than its counterparts at equal doses.

3.5.2 | Biodistribution study

Tumour localisation

As shown in Figure 5, the 100 nm formulation level in the tumour was significantly more than that of 200 and 400 nm formulations at 24 ($p < 0.05$) and 48 h ($p < 0.001$) post-treatment. In the Caelyx® treatment group, the Dox concentration in the tumour was similar to 100 nm formulation after 48 h, and it accumulated in the tumour more than 200 and

400 nm formulations ($p < 0.001$). In general, formulations with a smaller size (Caelyx® and 100 nm) were more successful in reaching the tumour sites due to their more prolonged blood circulation with a higher chance of penetration into the tumour tissues.

Non-tumoral tissues

The Dox concentration in blood was not detectable during this period, while the other formulations and Caelyx® indicated lengthier blood circulation time in vivo. Additionally, the 100 nm formulation showed longer circulation than 200 and 400 nm formulations. All the formulations followed a significant downward trend in the first 3 h, which decreased minimally over the next 48 h (Figure 6).

TABLE 3 Therapeutic efficacy data of different PEGylated nanoliposomal formulations, Caelyx® and free Dox in BALB/C mice bearing C26 tumour. Data represented as mean ± standard deviation ($n = 5$)

Treatments	TTE (days ± SD)	TGD (%)	MST (days)	ILS (%)
100 nm	41.90 ± 3.88	61.30	42.00	48.50
200 nm	39.00 ± 6.33	50.00	38.00	34.30
400 nm	33.50 ± 5.22	28.90	34.00	20.20
Caelyx®	50.00 ± 4.71	92.30	48.00	69.60
Dox HCl	32.00 ± 5.16	23.10	32.00	13.00
PBS	26.00 ± 2.72	-	28.30	-

Abbreviations: ILS, Increase in life span; MST, Median survival time; SD, Standard deviation; TGD, Tumour growth delay; TTE, Time to reach endpoint.

The liver and spleen of mice received nanoliposomal treatments (Caelyx®, 100 nm, 200 and 400 nm) showed higher Dox accumulation. To provide a detailed analysis, the amount of 200 and 400 nm formulations in the liver was notably greater than that of Dox, Caelyx®, and 100 nm formulation after 24 h ($p < 0.001$, $p < 0.01$, and $p < 0.01$, respectively). There was not a considerable difference between the amount of 400 nm and the other formulations in the liver after 48 h. In addition, Dox concentration of 200 and 400 nm formulations in the spleen was higher than that of 100 nm, Caelyx®, and Dox after 24 and 48 h post-injection. Increasing levels were statistically significant for 200 nm at 24 and 48 h ($p < 0.001$), and for 400 nm at these time points ($p < 0.0001$). Moreover, the 400 nm formulation concentration in the spleen was substantially higher than 200 nm after 48 h ($P < 0.05$).

None of the PEGylated liposomal formulations showed a significant difference in the heart. Unexpectedly, in the heart, drug concentration after 24 h was significantly higher than that after 48 h.

The 200 nm formulation showed the highest concentration in the kidney after the forgoing time period. Moreover, nanoparticles with a smaller size (100 nm) underwent renal clearance upon intravenous administration.

When it comes to lungs, nanoparticles with a bigger size (200 and 400 nm) accumulated more readily within the lungs' capillaries after the time points. In more detail, these amounts of increase were statistically significant for 200 nm formulation at 24 and 48 h ($p < 0.01$), and for 400 nm after 24 h ($p < 0.001$) in comparison with 100 nm formulation, Caelyx®, and Dox.

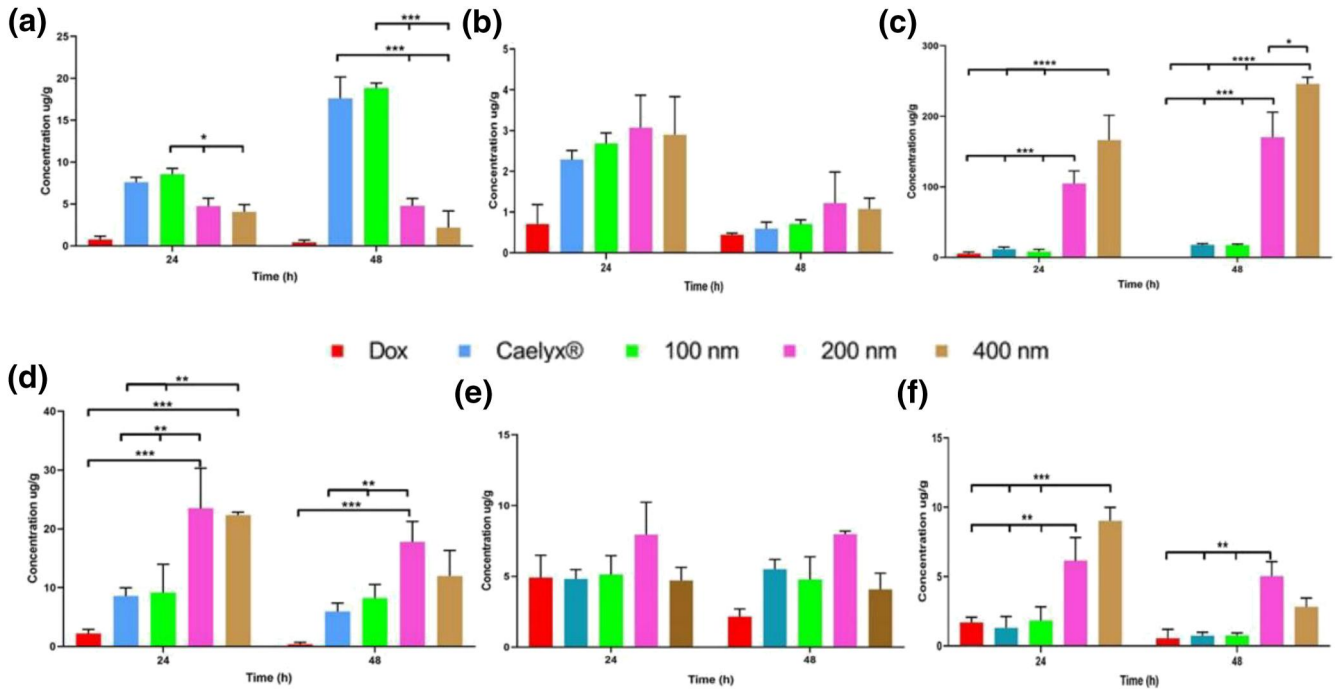

FIGURE 5 Biodistribution of different PEGylated nanoliposomal formulations, Caelyx® and free Dox in organs including (a) Tumour (b) heart, (c) spleen, (d) liver, (e) kidney and (f) lung in BALB/c mice bearing C26 tumour after a single dose of 10 mg/kg administered iv 14 days after the tumour inoculation. Data expressed as mean ± S.E.M. Statistically significant differences are shown as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.000.1$

3.5.3 | Pharmacokinetic study

The pharmacokinetic analysis was carried out using the non-compartmental model for the PEGylated nanoliposomal formulations, Caelyx®, and free Dox. The blood samples drawn from mice were taken at the aforementioned time points after intravenous bolus input. As shown in Table 4, the Caelyx® elimination half-life was significantly higher than that of the other PEGylated nanoliposomal formulations ($p < 0.0001$). There was also no statistically significant difference between 100 nm, 200 nm, and 400 nm formulations regarding half-life. Furthermore, an increase in the PEGylated nanoliposomal formulations' particle size resulted in a considerable AUC and AUMC decrease (from 1496.22 to 228.63, and from 61,400.57 to 2554.49, respectively). The PEGylated nanoliposomal formulations' clearance rate and distribution volume followed upward trends (0.011–0.043 and 0.244–0.923, respectively), with the particle size increasing from 100 to 400 nm. Moreover, Caelyx® showed the lowest clearance rate and volume distribution (0.005 and 0.161, respectively), while these amounts for Dox-free form were the highest in comparison with the other formulations (0.227 and 1.117, respectively). The Caelyx® MRT was substantially higher than that of the other PEGylated nanoliposomal formulations ($P < 0.0001$).

4 | DISCUSSION

In the current study, we developed and compared four different PLD sizes, including 90, 100, 200, and 400 nm regarding their antitumour activities after their passive accumulation in the tumour microenvironment via the EPR effect. According to previous studies, the most promising choice for drug delivery that has received the most research among the various types of NDDSs for cancer therapy is the liposomal drug delivery system. Additionally, to date, liposomal drugs form the largest number among different clinically approved nanopharmaceuticals. As is demonstrated in Table 5, liposomal drug delivery system offers various benefits, some of which are favourable pharmacokinetic features, protection from enzymatic degradation of the therapeutic substance, high encapsulation efficiency, and co-delivery of drugs. Moreover, the drug is encapsulated into liposomes to help reduce the unwanted adverse effects of frequently used chemotherapeutics, such as the cardiotoxicity that typically happens when anthracyclines are administered [34, 35].

The prepared-PLD particle sizes were 100 nm, 200 nm, and 400 nm, which were higher than Caelyx®. Several reports have documented that vesicles with 20–200 nm size range have the potential for successful accumulation in the tumour mass

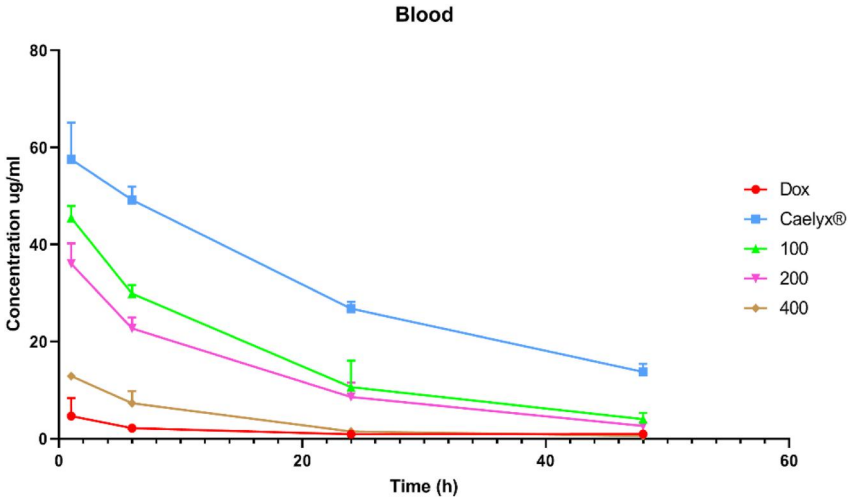

FIGURE 6 Biodistribution of different PEGylated nanoliposomal formulations, Caelyx® and free Dox at different time points in blood in BALB/c mice bearing C26 tumour after a single dose of 10 mg/kg administered iv 14 days after the tumour inoculation. Data expressed as mean ± SEM

TABLE 4 The plasma pharmacokinetic parameters using non-compartmental methods in BALB/c mice bearing C26 tumour following intravenous injection of free Dox, Caelyx® and different sizes of PEGylated nanoliposomal formulations at a single dose of 10 mg/kg

Formulations	$AUC^{a}_{0\text{-}t}$ (μg/ml*h)	$AUMC^{b}$ (μg/ml*h^2)	MRT^{c} (h)	Cl ((mg)/(μg/ml)/h)	V ((mg)/(μg/ml))	$T_{1/2}$ (h)
Dox	43.97	216.18	4.91	0.227	1.117	3.84
Caelyx®	1496.22	61,400.57	31.53	0.005	0.161	22.67
100 nm	861.56	15,726.41	18.25	0.011	0.244	14.61
200 nm	653.09	11,026.80	16.88	0.015	0.299	13.56
400 nm	228.63	2554.49	19.72	0.043	0.923	14.63

[a]stands for the area under the curve.

[b]stands for the area under the first-moment curve.

[c]stands for MRT.

TABLE 5 Nanocarriers used as drug delivery systems

NDDSs	Advantages	Disadvantages	Ref.
Liposome	• 25–1000 nm	• Short half-life	• [58, 59]
	• Suitable for delivery of various drugs and molecules	• poor stability	
	• Surface functionalisation	• Low circulation time without surface modification	
	• Targetability	• Possibility of phospholipid oxidation	
	• High drug loading capacity		
	• Biocompatibility		
	• Biodegradability		
	• EPR mechanism		
	• Dual targeting		
	• Combination therapy		
	• Overcome the toxicity of the encapsulated drugs		
Dendrimer	• 1–10 nm	• Complex design	• [60, 61]
	• Uniform structures	• Toxicity	
	• Enhanced therapeutic efficacy	• High clearance by RES	
	• Suitable for gene delivery		
	• Dual targeting		
Inorganic nanomaterials	• High surface area	• Hard degradability	• [62]
	• Facile preparation	• Low hydrophilicity	
	• Size stabilisation	• Rapid blood clearance by RES	
		• Toxicity	
Solid lipid nanoparticles	• 10–1000 nm	• Considerable clearance by RES	• [63, 64]
	• Biocompatibility	• Not applicable to hydrophilic drugs	
	• Biodegradability		
	• Low toxicity		
	• Surface functionalisation		
Polymeric nanoparticles	• 1–1000 nm	• Toxic degradation process	• [65]
	• Biodegradability	• Immunological response due to degradation rate	
	• Stability		
	• Surface functionalisation		
Micelles	• 10–100 nm	• Immunological response	• [66]
	• Suitable for both lipophilic and hydrophilic compounds	• poor stability in vivo	
		• Dissociate below Critical Micelle Concentration (CMC)	

and inflammatory sites [36]. The liposomal formulations had PDI of 0.166, 0.103, and 0.206 for 100 nm, 200 nm, and 400 nm, respectively, which were all within the suitable range of pharmaceutical products, and demonstrated uniform distribution in all treatments [37].

The surface charge has an undeniable role in protein adsorption and influences tissue distribution of the liposomes [38]. Many studies have shown that negative and neutral charged liposomes have lengthier circulating half-lives with suitable pharmacokinetic profiles [39]. Yamamoto et al. showed the benefit of neutral and anionic polymer micelle surfaces for long circulation and illustrated that negatively charged nanoparticles bring about lower liver and spleen accumulation [40]. We, therefore, prepared all liposomal formulations in this study, with a negative charge of about −17 mV. Intriguingly, there was not any significant difference between the Caelyx® surface charge (Zeta potential: −17.2 mV), and 100, 200, and 400 nm PLDs (Zeta potentials: −17.0 mV, −17.8 mV, −17.3 mV, respectively).

The remote loading method was utilised to encapsulate Dox into liposomes (Fritze et al., 2006). One of the prominent findings was the results of Dox content (1.9, 1.7, and 1.6 mg/ml Dox entrapment) and encapsulation efficiency (99%, 98%, and

87% EE) for 100 nm, 200 nm, and 400 nm PLDs, respectively. This efficient drug loading could be related to successful remote loading and Dox encapsulation into the liposomes. These findings are in agreement with the previous investigations, showing that in the large majority of cases, remote loading is the best method for reaching active ingredients' high encapsulation into the liposome [41, 42].

Measuring localised liposomes drug release at the affected sites allowed us to understand the drug mechanism behaviour and predict the in vivo formulations' efficacy [43]. As demonstrated in Figure 2, the plasma release showed upward trends in the first 6 h and a minimal increase over the next hours for all formulations. Furthermore, the drug release was assessed under several pHs of 5.5, 6.5, and 7.4 to imitate the endosomal, tumoral, and physiological fluid release, respectively [44, 45]. More precisely, drug release increased minimally in all prepared formulations during the investigational time in different conditions. In general, there was not any considerable difference among all PLDs with different release profiles.

Cholesterol and HSPC found in all Dox formulations were used to control drug release during blood circulation and increase the longevity and stability of the liposomal bilayer [46]. This result is in line with the study findings that indicated the influence of the liposomal lipid compositions on the drug release behaviour from nanoliposomes [47, 48]. Our data suggest that particle size could not make any significant difference in the release profile.

The cytotoxicity of the prepared formulations against C26 (colon carcinoma) cell line was measured. Dox toxicity could be decreased by producing Dox liposomal formulations, which can limit the normal tissue uptake, improve therapeutic index, and decrease adverse effects [49]. Free Dox had greater toxicity than liposomal Dox because it freely passes through the cell membrane and exerts its cytotoxic effect. However, the higher IC50 of PLDs might be related to the electrostatic repulsion between the cancer cells and anionic liposomes' negative surface charge, leading to lower Dox toxicity [50]. Our findings showed that there is not any significant difference in toxicity between the Caelyx® and other PLDs. Additionally, this study showed that particle size does not affect cytotoxicity tests in *in vitro* studies. There was no considerable difference in cell interaction, IC50, or release profile in different pH conditions when using PLDs for delivering doxorubicin. Moreover, the Dox fluorescence properties were used to assess the C26 cell line uptake in flow cytometry tests. We demonstrated that not only all the PLDs showed similar cellular uptake profiles but also similar toxicity and release profiles.

The in vivo anti-tumour activity of the nanoliposomal formulations was assessed to determine the therapeutic efficacy in animal models. In our experiments, the formulations' efficiencies were compared with PBS-treated mice as the control group. The graphs for tumour volume represent the mean tumour size in different groups versus time, which depict that the effect of 100 nm formulation on the tumour growth rate was similar to Caelyx®. Weight reduction profiles in BALB/c mice body masses for all formulations (10 mg/kg) were similar compared to Caelyx®, meaning that the treatments did not produce any apparent toxicity. Furthermore, the survival results are shown as Kaplan-Meier plots. The mice which were treated with Caelyx® exhibited a significantly longer survival with a higher therapeutic effect in comparison to 100 nm, 200 nm, and 400 nm PLDs. Other factors related to the therapeutic efficacy, namely %TGD, MST, TTE, and Increase in life span were measured, which are correlated with the Dox formulations release profile. The results also indicated higher therapeutic efficacies for all liposomal formulations than PBS or free Dox. Levels of toxicity and animals' life span in this investigation were in the following order: Caelyx® > 100 nm > 200 nm > 400 nm.

The biodistribution results demonstrated considerable differences between Caelyx®, 100 nm, 200 nm, and 400 nm liposomal formulations and the control group. The liposomal formulations with a smaller size (Caelyx® and 100 nm) showed longer blood circulation time and improved pharmacokinetic profiles. This result is in line with Blanco and his co-workers' findings, which have indicated that liposomes averaging ~100 nm showed prolonged half-lives in the blood circulation and a higher tendency to extravasate through the tumours vascular network [51].

Moreover, Caelyx® and 100 nm formulation tend to accumulate in tumour tissue much more than they do in other tissues due to the EPR effect. On the other hand, larger particles, 200 and 400 nm PLDs, did not show any evident tendency to localise in tumour tissue. The EPR mechanism causes smaller particles accumulate in tumour tissue, and this localisation due to their extravasation through leaky vasculature works very well for tumour tissues [52, 53]. These findings are in agreement with the Zein *et al*. results in 2020, which have indicated that the best tumour accumulation due to the EPR effect needs a nanoparticle size between 100 and 150 nm [54]. However, the comparison between Caelyx® and 100 nm PLD suggested that by reducing PLDs sizes lower than 100 nm, the tumour accumulation increased.

According to the results from this study, Dox accumulated more in the liver and spleen of PLD treated animals than those treated with the conventional form of Dox. Moreover, at 24- and 48-h post-injection, the number of larger nanoparticles (200 and 400 nm) in the liver and spleen was higher than that of Caelyx® and 100 nm formulations. Larger particles are more rapidly taken up by the liver and spleen, and entrapped in these organs' reticuloendothelial system (RES), leading to shorter blood circulation [55]. There was no statistically significant difference in drug accumulation in the heart tissue among different PLDs, suggesting that the drug delivery to the heart did not depend on the formulations' particle size. It was also shown that the 200 nm formulation had the highest concentration in the kidney after the forgoing periods. This could be attributed to probable 200 nm formulation aggregations. Moreover, nanoparticles with a bigger size (200 and 400 nm) accumulated more readily within the lungs' capillaries after these time points. The pharmacokinetic study also proved that an increase in particle size causes better detection by the RES and results in faster blood clearance of the formulations [56]. Additionally, Caelyx® showed the highest elimination half-life,

leading to higher stability, longer blood circulation, and higher accumulation in tumour sites [57].

Even though there are several investigations concerning the effect of liposome size on the therapeutic efficacy, biodistribution, release, and toxicity profile of PLD, none have provided a comprehensive and a well-organised overview. Therefore, we conducted this study to comprehensively provide an overview regarding the importance of vesicle size in the delivery of drugs to the tumour microenvironment for the treatment of cancer.

5 | CONCLUSION

In this study, the in vitro and in vivo impact of PLD size were evaluated. Our data looking into 90, 100, 200, and 400 nm PLD sizes revealed no considerable effect on the in vitro release, cell uptake, and cytotoxicity of the formulations. However, the data indicated that particle size affects biodistribution and therapeutic efficacy. According to our results, Caelyx® and 100 nm formulations demonstrated the highest C26 tumour accumulation compared to the other formulations. Caelyx® showed better therapeutic efficacy as it could increase mice longevity. It could also delay tumour growth and lengthen the survival time compared to the other PEGylated nanoliposomal formulations and Dox conventional form. Therefore, Caelyx® (90 nm particle size) showed the most appropriate plasma profile, which resulted in the highest survival rate in vivo, in comparison with 100, 200, and 400 nm formulations. The study shows the importance of vesicle size in the targeting of nanoparticles to the tumour microenvironment for cancer treatment. More studies on the efficacy of size in various tumour types should be performed to obtain a more comprehensive conclusion.

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CONFLICT OF INTEREST

All authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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