

Supplemental Information

Role of Physicochemical Characteristics in Liposomal Doxorubicin: Effects on Pharmacokinetics and Biodistribution in Tumor-Bearing Mice

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S-1 Characterization of the Liposomal Formulations

Table S-1 Summary of Certification Data for the Formulations Provided by FormaMax Scientific Inc.

Formulation	100 nm	150 nm	250 nm
Lipid Composition	HSPC/CHOL/mPEG2000-DSPE (56.3:38.4:5.3 mol%, 3:1:1 wt/wt)		
Active Drug	Doxorubicin HCl	Doxorubicin HCl	Doxorubicin HCl
Lipid Concentration ^a	16.0 ± 0.5 mg/mL	16.0 ± 0.5 mg/mL	16.0 ± 0.5 mg/mL
Drug Concentration ^a	2.00 ± 0.02 mg/mL	2.00 ± 0.02 mg/mL	2.00 ± 0.02 mg/mL
Free Drug Concentration	0.02 mg/mL	0.02 mg/mL	0.05 mg/mL
Drug Encapsulation Efficiency	99.0%	99.0%	97.3%
Hydration Solution	250 mM (NH ₄) ₂ SO ₄	250 mM (NH ₄) ₂ SO ₄	250 mM (NH ₄) ₂ SO ₄
Osmolarity	348 ± 2 (mOsmo)	342 ± 2 (mOsmo)	353 ± 2 (mOsmo)
Effective Particle Size (DLS, H ₂ O)	101.3 nm	155.3 nm	258.6 nm
Particle Size (DLS, vol weighted)	97.1 ± 1.0 nm	138.4 ± 1 nm	217.0 ± 1.0 nm
Half-width	21.9 ± 0.11	35.0 ± 5.7 nm	85 ± 10 nm
Polydispersity	0.05 ± 0.01	0.05 ± 0.02	0.11 ± 0.03
External Buffer Solution	10% (w/v) Sucrose, 10 mM Histidine, pH 6.6		

^aMeasured by uv spectrophotometry. HSPC: fully hydrogenated phosphatidylcholine (Lipoid), CHOL: cholesterol (Lipoid) mPEG2000-DSPE: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (Lipoid).

Table S-2. Characterization of doxorubicin-loaded liposomes.

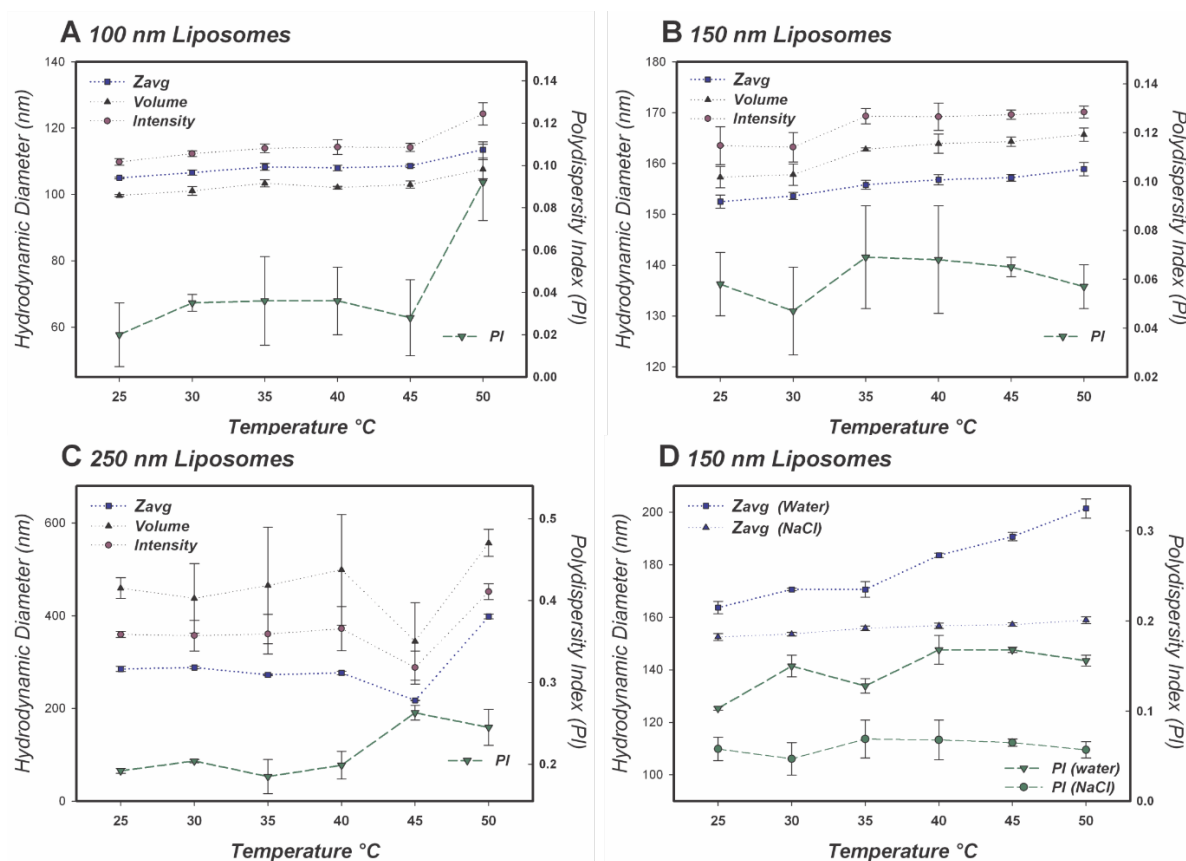
Nominal Size (nm)	100	150	250
Initial Values:			
Zavg (nm)	106.1	154.4	260.3
Polydispersity Index (PI)	0.036 ± 0.001	0.044 ± 0.012	0.182 ± 0.001
Intensity	111.2 ± 0.1	164.6 ± 1.9	318.6 ± 6.4
D10(i)	79.0	112.3	155.7
D50(i)	108.3	158.7	289.7
D90(i)	150.3	224.7	523.0
Span (i)	0.658	0.708	1.269
Volume	101.0 ± 1.2	158.9 ± 1.9	368.3 ± 15.0
D10(v)	70.2	103.7	137.7
D50(v)	96.7	152.0	344.3
D90(v)	139.3	228.0	633.7
Span (v)	0.715	0.818	1.441
Zeta Potential (mV)	-9.30	-14.80	-11.67
Target Encapsulation	>95%	>95%	>95%
Observed % Free Doxorubicin	1.94 ± 0.37 %	2.32 ± 0.01 %	6.08 ± 1.97 %
Percent Irregular Shape*	6.89	12.02	58.10
After 7 months:			
Zavg (nm)	104.6	150.9	260.8
Polydispersity Index (PI)	0.036 ± 0.018	0.061 ± 0.012	0.180 ± 0.007
Intensity	110.1 ± 0.8	162.7 ± 1.2	327.2 ± 9.5
D10(i)	77.3	107.0	158.0
D50(i)	107.0	156.3	297.3
D90(i)	150.3	231.7	549.0
Span (i)	0.682	0.797	1.314
Volume	99.2 ± 1.2	155.5 ± 0.5	388.2 ± 30.6
D10(v)	65.9	95.6	149.0
D50(v)	94.6	145.7	363.0
D90(v)	138.3	233.3	669.7
Span (v)	0.740	0.946	1.434
Zeta Potential (mV)	-11.80	-25.33	-14.73

For DLS measurements, samples were diluted 100 x into 10 mM NaCl and run on a Malvern Zetasizer Nano ZS at 25°C. *Estimated by manually counting regular and abnormal liposomes on TEM micrographs.

Liposome thermal stability

Shelf-life stability was monitored by DLS for size and zeta potential over the duration of *in vivo* study (<7 months) for each individual liposome formulation. Thermal stability was also performed upon arrival of the individual liposomal formulations. Liposomal formulations were diluted 1:1000 in 10mM NaCl, and size was continuously monitored by DLS over a temperature range of 25 – 50 °C in 5 °C increments. The DLS software adjusted the temperature in the sample holder, while a thermal cap maintained it. A four-minute delay allowed time for the sample to equilibrate prior to measurements (Figure S-1). All three

formulations remained stable up to 40°C, while increases in size and polydispersity occurred in certain cases at temperatures above 40°C. It was noted that the liposomes were less stable when diluted in water than when diluted with 10 mM NaCl (S-1D). Additionally, liposomal sizes were monitored following manipulation with various gauge syringe needles, which were used to aliquot or intravenously (IV) inject liposomes, to ensure size integrity under experimental conditions (data not shown).



Supplemental Figure S-1. Effect of temperature on liposome stability. Nominal A) 100 nm B) 150 nm and C) 250 nm formulations continuously monitored by DLS for changes in hydrodynamic size with increasing temperature. D changes in size and stability when water is used as diluent.

S-2 Separation of Free and Encapsulated Doxorubicin

Introduction

The relative percentage of unencapsulated doxorubicin observed across times in the PK study consistently indicated an approximate 1-2% free drug component. To investigate whether this was true or an artifact of the method, free drug was separated from liposomal drug by either gel filtration or solid-phase extraction (SPE), and then the resultant encapsulated liposomal fraction was rerun on SPE to determine the subsequent encapsulated and free drug fraction.

Methods

Free drug evaluation following gel filtration or solid-phase extraction

25 μ L of liposomal doxorubicin (Doxil®, Janssen Pharma) passed through a PD-10 desalting column (GE Healthcare) using sucrose/histidine buffer as the mobile phase, and the liposomal drug band was collected in a vial with 50 μ L of plasma in it (~500 μ L). A 25 μ L sample of stock drug diluted in 5% dextrose/50 μ L plasma (500 μ L) was used as a comparative control for SPE. An internal standard was added to the fixed volume of sample and followed by SPE loading. Liposomal fractions were collected for both samples. Following gel filtration, there should be no free drug present in the sample. An additional test was done in which the drug stock was loaded onto the SPE cartridge, the encapsulated fraction was collected, and then reloaded onto a new SPE cartridge to check the amount of free drug.

Liquid Chromatography and Mass Spectrometry of Doxorubicin

Separations were performed using an Agilent 1290 series UPLC, iFunnel 6550 QTOF with an Accurcore C18 column with a methanol/formic acid (0.1%) gradient to water with 0.1% formic acid, running at 400 μ L/min for the pump. Daunorubicin (Sigma-Aldrich) served as an internal standard. Calibration curves were made from serial dilutions of 25 - 400 ng/mL. Further details are found in the methods section of the manuscript.

Results & Conclusions

Sample	Free %
No PD-10 prior to SPE	1.8
PD-10 prior to SPE	1.5
SPE liposomal fraction rerun on SPE	1.3

Supplement Table S-3. Percentage of unencapsulated doxorubicin following gel filtration or solid phase extraction.

The sample that was processed as in the PK study without PD-10 treatment prior to SPE indicated 1.8% unencapsulated drug (Table S-3). After PD-10 treatment, free drug should have been removed through size exclusion, yet 1.5% of free drug was still present in the sample. The liposomal fraction collected after SPE and run again on SPE showed 1.3% unencapsulated drug. It is likely that this freshly released free doxorubicin is predominantly the result of normal release via the equilibria between soluble doxorubicin and precipitated gelatinous doxorubicin sulfate within the liposomes and diffusion of doxorubicin across the liposomal membrane [1, 2]. However, structural stress on the liposome may result in degradation of liposomal integrity and subsequent loss of internal sulfate, thereby increasing the solubility of the doxorubicin sulfate precipitate and subsequent release. Simply increasing the pressure conditions of SPE

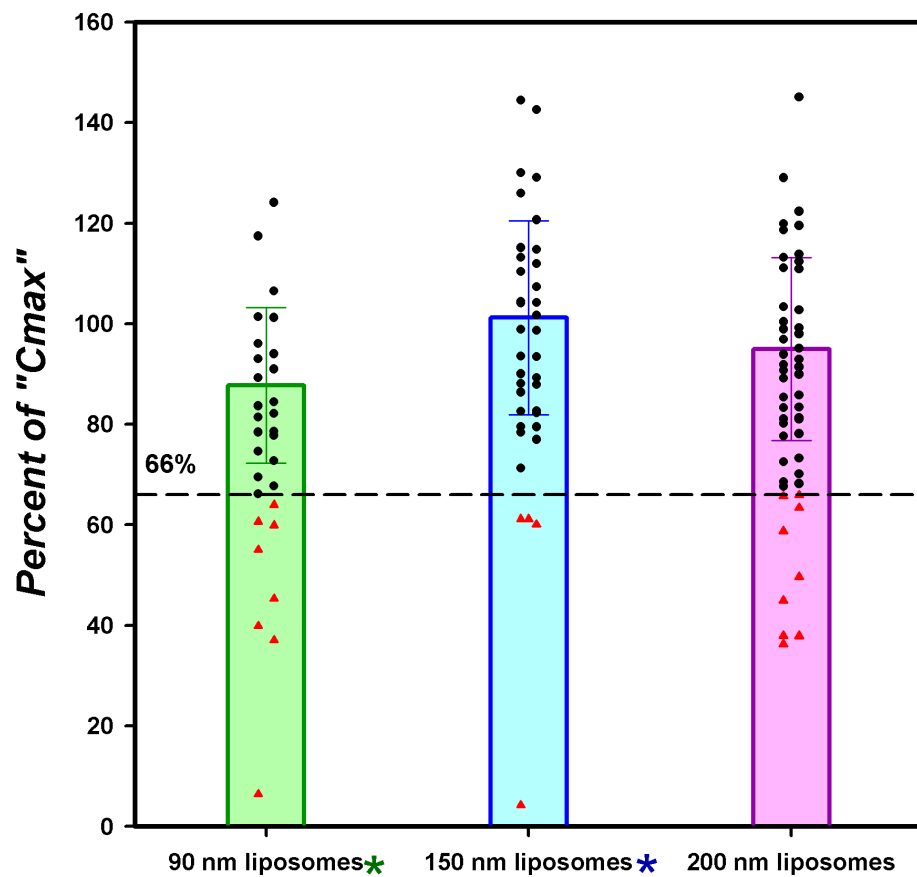
can dramatically result in higher free drug recovery. In addition, user variability was also observed; all further evidence that the SPE procedure played a significant role in the unencapsulated drug observed.

S-3 Evaluation of injection success

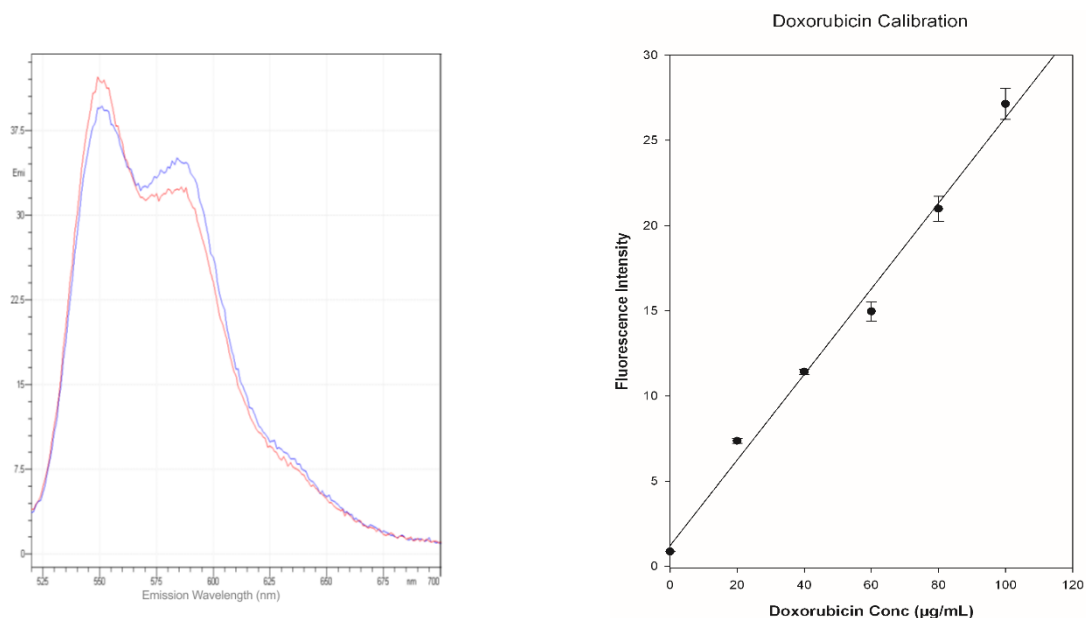
Accurate injection of liposomal nanoparticles into the tail veins of female NU/J mice requires significant skill and dexterity due to the small size of the animal and the requirement for the internal diameter of the injection needle to allow passage of the liposomal suspension without restrictions or vortices that could damage or deform the liposomes [3]. During the initial part of the study (using 30 gauge, 0.5 inch, 0.3 ml insulin syringes), it was observed that a small proportion of the dosed mice had lower than expected serum doxorubicin concentrations at 15 minutes and 1 hour after dosing, suggesting that none or only a small amount of the administered dose had entered and remained within the tail vein, while the majority of the dose extravasated within the tail tissue. The technicians conducting the dosing procedure were not always able to establish whether the dosing had been successful. While this type of undetected dosing error may be incorporated into the experimental variation of typical efficacy studies where large differences are expected between the test article and control, they would limit the statistical power to detect small differences between similar test articles as is required for this study. Therefore, the following method was developed to recognize and eliminate partially dosed mice.

In subsequent animals, a 5 μ L blood sample was collected 20 minutes after dosing and assayed for doxorubicin concentration by a rapid fluorescence spectrophotometric procedure described below. A 5 μ L aliquot of blood was also collected from the cardiac blood samples of animals sacrificed at the 15-minute time point. The observed doxorubicin concentration was compared with a theoretical $C_{\max}$ concentration calculated from the injected dose and a previously published total blood volume estimate for female mice [95 mL/kg] [4]. Observed values (Figure S2) ranged from < 10% to > 150% of this value. Values >100% of $C_{\max}$ are possible because the published blood volume was determined using the dilution of labeled albumin to measure the serum volume component. Albumin will freely pass into more of the interstitial space than 100 – 250 nm liposomes and will effectively measure both the total vascular and interstitial serum volume. The very low 20 min blood doxorubicin concentrations in some of the samples implied that insufficient dose had entered or remained in the vein of these mice. An arbitrary value of 66% of apparent $C_{\max}$ was used to eliminate mice that possibly received an insufficient dose (Fig S2). The majority of the mice dosed with the three liposomal preparations gave values above 66% and were included in the study. While there were no significant differences between the preparations and the proportion of mice that failed dosing, the frequency of failed dosing did appear to decrease during the course of the study as the technicians gained more experience in dosing the animals. Technicians had to practice with “red dextran” and be certified prior to participating in the study to limit insufficient dosing. The overall mean percentage of the successful mice in the 100 nm group was significantly lower than that for the 150 nm group. This may be due to more of the smaller liposomes entering the interstitial space than larger ones.

The following is a summary of the results of fluorescence spectroscopy performed on nominal 100, 150, and 250 nm liposomes. A standard dose range of 100% - 20% of apparent $C_{\max}$ was used for calibration. Twenty minutes post-IV injection, blood was collected and analyzed to validate a successful injection.



Supplemental Figure S-2. Percent of theoretical C_{max} of liposomal doxorubicin preparations measured in blood samples taken 20 mins after dosing. Bars represent mean $\pm$ SD of accepted mice. (Individual values shown as black circles; values for excluded mice shown as red triangles. *denotes significantly different ($P < 0.05$) from the correspondingly marked group (means of included mice only) with Tukey's range test.



Supplemental Figure S-3. Fluorescence spectroscopy nominal 100 and 250 nm liposomes (left) and calibration curve (right). Fluorescence spectra of disrupted doxorubicin-loaded liposomes (blue = 100 nm, red = 250 nm liposomes) at theoretical C_{max} (100% in blood of mouse) tested in Sheep's Alservers blood. Calibration of fluorescence used Alservers blood spiked with doxorubicin.

Synopsis of Method:

1. The lateral saphenous vein is punctured with a lancet.
2. 5 µL of blood is collected in a 5 µL capillary tube and placed in a 1.5 mL microfuge tube.
3. With a 27G 1 mL syringe, 1.0 mL of acetonitrile is passed through the capillary tube to remove the blood sample and break open the liposomes. Then 2.5 µL of 1 N HCl is added to the tube to assist with the solubility of the doxorubicin.
4. The sample is vortexed and then spun in a centrifuge at 10,000g for 5 min at 15°C.
5. While the samples are spinning, 1.6 mL of acetonitrile is added to a 13 x 100 glass tube. Once the blood/cell fragments are spun down, 800 µL of the supernatant from the sample is added to the 1.6 mL of acetonitrile in the glass tube.
6. Gently vortex the glass tube prior to adding to a fluorescence cuvette for reading.
7. Read the fluorescence spectrum: Ex 509 nm, emission 520–700 nm; record the maximum peak at approx. 560 nm under these conditions.
8. Shimadzu settings: EXT slit 5 nm, Em slit width 5 nm, high sensitivity.
9. Between samples, wash the cuvette with deionized H₂O and methanol.

Accuracy of Analysis: QAQC methods/results that validate analyses.

Based on data in the literature, a 1 mL total blood volume in mice was assumed for QAQC of the method. A standard dose range of 20 – 100 µg/mL doxorubicin free base derived from a stock solution of doxorubicin HCl corresponding to approximately 20 -100% of apparent C_{max} was used for calibration. The standards were prepared in sheep's blood (Alser's solution) from dilutions of a stock (10 mg/ml) stock solution. These spiked blood samples were then processed by the same procedure as listed in the “synopsis of method,” with the following exception. Blood collected in the capillary tube listed in #2 above comes from the standard solutions and not from bleeding of mice. From the determined fluorescence curve for the standards, a cutoff criterion (66% of C_{max}) was implemented for inclusion/exclusion of experimental mouse samples. This 66% cutoff value was chosen arbitrarily prior to sampling the mice and was 1.4, 1.8 and 1.6 standard deviations below the mean percentage values for the 100, 150 and 250 nm dose groups, respectively (Figure S-2).

S-4 Estimation of residual blood in tissues

Table S-4. Residual blood in tissues

TISSUE	BLOOD/TISSUE (µL/g) mean ± SEM (N = 6)
Liver	6.96 ± 0.67
Lung	49.38 ± 4.22
Heart	6.75 ± 1.18
Kidney	5.22 ± 0.27
Tumor	1.60 ± 0.50

Because the cardiac puncture procedure used for blood collection was not expected to completely exsanguinate the mouse, residual blood was estimated by measuring hemoglobin content in the tissue homogenates and comparing the hemoglobin concentration in a blood sample from the same animal. The assay was used with a subset of mice, and the values were given as µL/g tissue to adjust tissue doxorubicin concentrations for each tissue. A 10 µL aliquot of fresh blood was diluted in water to lyse the erythrocytes and stored for up to 2 weeks at -20°C. Excess tissue homogenate (400 – 600 µL) from the sample preparations was stored with the blood samples. On the day of analysis, the samples were thawed on ice. Blood samples were diluted 100-fold in 100 mM potassium phosphate buffer, pH 7.4, and tissue homogenates were diluted 12-fold in the same buffer. Hemoglobin was estimated spectrally as carboxyhemoglobin by the method of Estabrook and Werringloer [5] using a Shimadzu UV-2550 double beam spectrophotometer. The amount of residual blood was calculated as µL/g tissue from the ratio of tissue and blood hemoglobin concentrations. Only lung tissue contained substantial volumes of residual blood (Table S-4). Spleen could not be evaluated by this method because of the high levels of endogenous hemoglobin present in this tissue. These values were used, together with the serum doxorubicin concentrations, to adjust measured tissue doxorubicin concentrations to eliminate the contribution of residual blood.

S-5 Doxorubicin concentration ratios

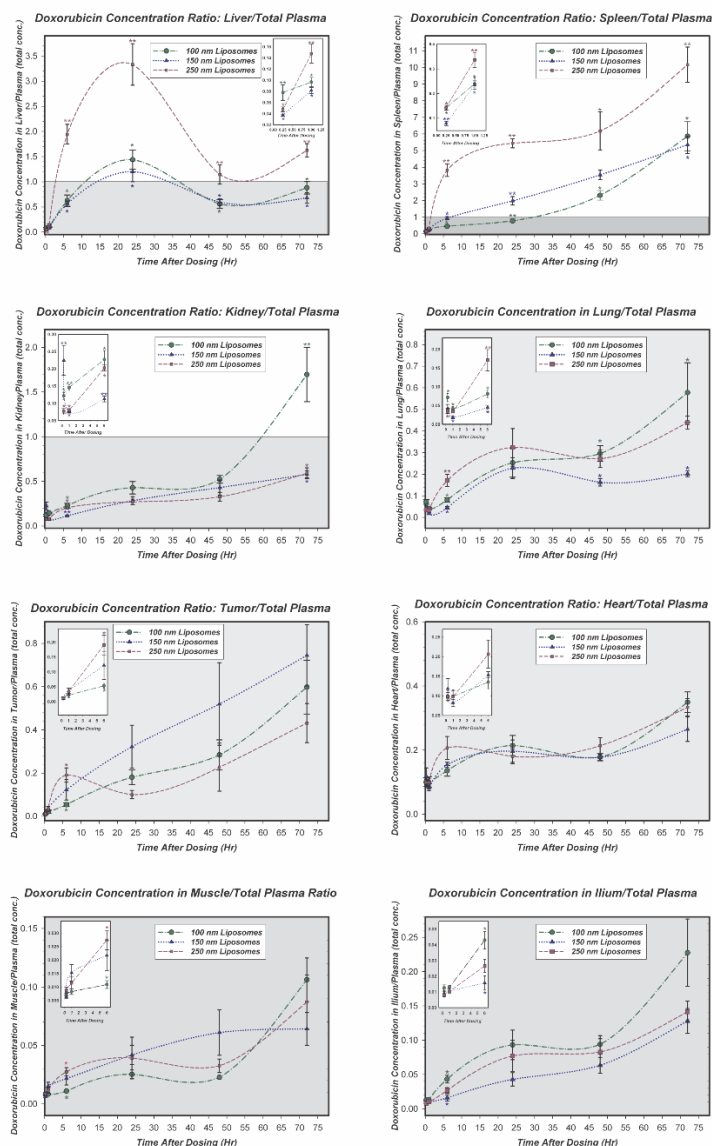


Figure S-4. Tissue per blood concentration ratios for doxorubicin. Graph points, given as mean $\pm$ SEM, were calculated from corresponding plasma total doxorubicin and tissue doxorubicin concentration values. Inset graphs enlarge the first 3 time points so that marked statistical significance can be visualized. *Significantly different ($P < 0.05$) from one other marked liposomal dose group, **significantly different ($P < 0.05$) from both other liposomal dose groups assessed using a Tukey's range test. Shaded areas on the main graphs denote values of < 1.0 meaning that the internal tissue concentration was less than the blood concentration. Doxorubicin only accumulates in the liver and spleen, and in kidney at 72 hours after dosing in mice receiving the 100 nm liposomes. Differences between liposome formulations in the other tissues are small and rarely statistically significant.

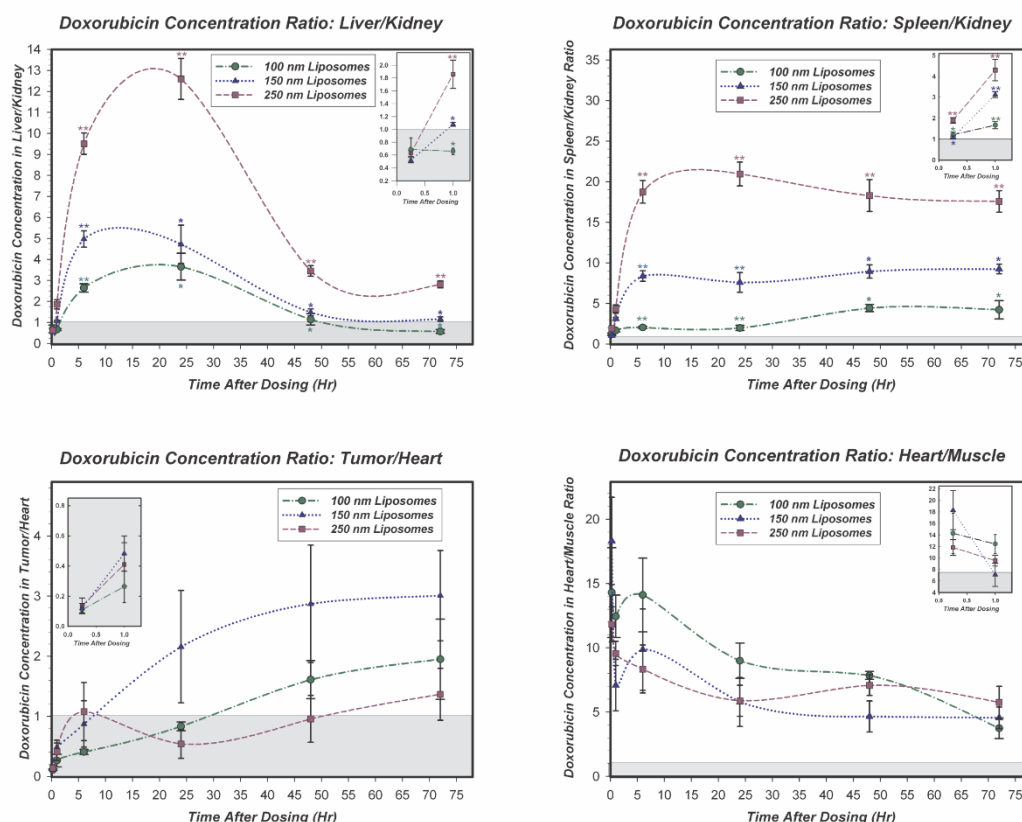


Figure S-5 Doxorubicin concentration ratios between different tissues. Graph points, given as mean $\pm$ SEM, were calculated from corresponding tissue doxorubicin concentration values. Inset graphs enlarge the first 2 time-points so that marked statistical significance can be visualized. *Significantly different ($P < 0.05$) from one other marked liposomal dose group, **significantly different ($P < 0.05$) from both other liposomal dose groups assessed using a Tukey's range test.

Clear, size-related, statistically significant differences in extractable doxorubicin concentrations are observed between the liposome formulations in liver relative to kidney between 1 and 24 hours and in spleen relative to kidney at all time points (Figure S-5). When extractable doxorubicin concentrations are compared between the tumor (target tissue) and heart (site of potential toxicity), there are no statistically significant differences between formulations at any time point. During the first 6 hours, greater concentrations of extractable doxorubicin accumulate in the heart than in the tumor, but by 48 hours more had accumulated in the tumor. Heart muscle accumulated considerably greater concentrations of extractable doxorubicin than skeletal muscle, possibly due to the high blood flow through the heart. There were no significant differences between the liposomal formulations for either the tumor/heart or heart/muscle ratios.

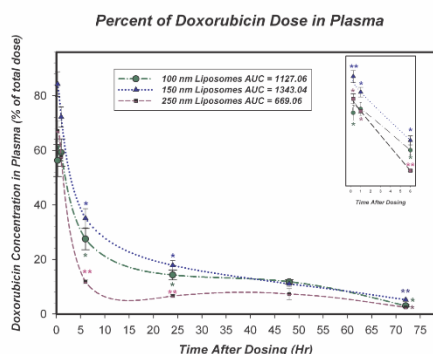
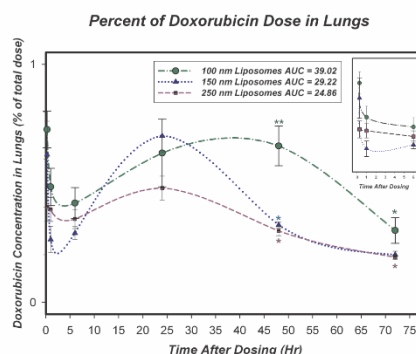
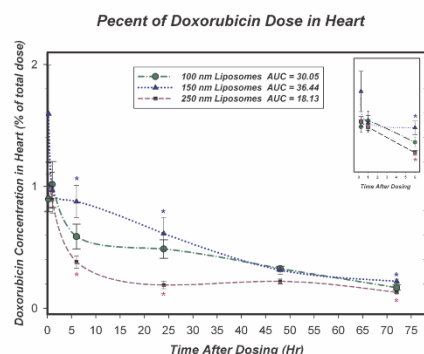
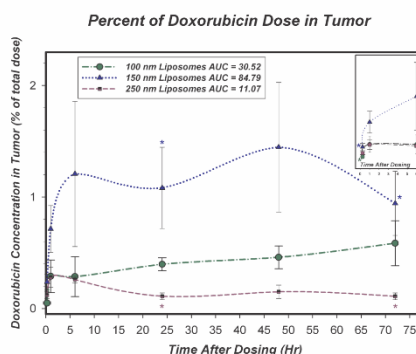
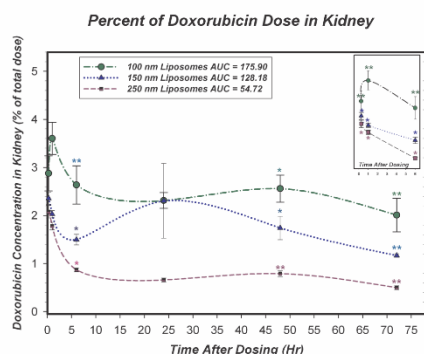
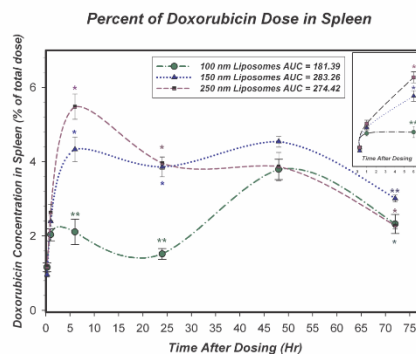
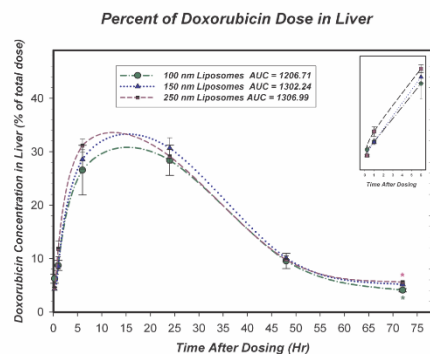


Figure S-6 The total doxorubicin content of each tissue/organ was calculated and plotted as % of total dose using individual concentration, organ and body weight values from individual mice. AUC values of each curve were used to calculate the % Delivery/exposure efficiency values shown in Table 4. [AUC/72 hr exposure time]

The method used here to determine %Delivery/Exposure Efficiency differed from that used by Wilhelm et al [6] in that data for individual tumor and body weights were available to use instead of mean or inferred values.

S-6 Evaluation of Extraction of Doxorubicin Intercalated with Calf Thymus DNA.

The observed low (66%) recovery of doxorubicin from spleen tissue suggested that the methanol extraction method used in this study might not have efficiently extracted doxorubicin that was intercalated to DNA. The spleen contains large amounts of extranuclear DNA derived from processing apoptotic cell debris [7]. Other researchers have reported incomplete extraction of intercalated doxorubicin from DNA [8, 9]. Two different general procedures have been used to maximize doxorubicin recovery from cells and tissues: (i) enzymatic digestion with proteases and DNase [8, 9, 10], or (ii) denaturation with acidified alcohols, chloroform, or acetonitrile [11, 12, 13, 14, 15, 16]. While the digestion procedure ensures that all intercalated doxorubicin will be released from the DNA, artifactual metabolism or oxidation of doxorubicin may occur during the enzyme incubations [9, 11].

The acidified methanol-based denaturation procedure developed for this study gave apparent recoveries of between 89 and 100% for tissues other than spleen, when spiked doxorubicin was extracted from homogenized tissue samples with methanol acidified to 1% acetic acid. Prior to centrifugation, the denatured tissue suspension was kept at -80°C for 1 hr to allow equilibration of the internal standard with any doxorubicin still bound to tissue fragments. Similar procedures have been reported to improve extraction efficiency [11, 15, 17]. However, quantification of all these denaturation methods is dependent on the assumption that the doxorubicin spike or internal standard spike behaves in an identical way to the doxorubicin in the homogenized tissue sample. Most tissue homogenization procedures leave the nuclear membrane intact, and evidence suggests that it provides a barrier to simple diffusion of anthracyclines, thus preventing immediate intercalation with nuclear DNA. In vivo, anthracyclines enter the nucleus predominantly by active transport mechanisms [18].

Subsequent to the work reported in the study, the ability of acidified methanol to extract intercalated doxorubicin from DNA was tested using purified calf thymus DNA (Millipore-Sigma). In the first experiment, acidified methanol was compared with acidified acetonitrile as used by Song et al. [12] and with 100% isopropanol. When added to calf thymus DNA dissolved in 10 mM Tris HCl buffer (pH 8.0) containing 2 mg/mL bovine serum albumin, doxorubicin rapidly intercalated with the DNA, resulting in reduced fluorescence. As shown in Table S-3, in all cases where DNA was present, the solvent extraction recovered <35% of the intercalated doxorubicin in the supernatant fraction following centrifugation. DNase digestion was required for complete recovery of doxorubicin from the sedimented DNA pellets. If DNA was omitted from the mixtures, 100% of the doxorubicin was recovered in the supernatant fractions, with all three solvents showing that they were able to extract doxorubicin that was bound to albumin. It was also noted that the doxorubicin emission spectra were dependent on the solvent used, which complicates exact quantification by fluorescence (Figure S-7) and required individual standard curves to be constructed for each solvent.

Table S-5 Efficiency of Solvent extraction in recovering doxorubicin intercalated to Calf Thymus DNA

Tris BSA Buffer	1,000 μ L	1,200 μ L	1,000 μ L	1,200 μ L	1,000 μ L	1,200 μ L
200 μ L DNA (mg) [μ mol]	0.528 [1.6]	-	0.528 [1.6]	-	0.528 [1.6]	-
4 μ L Doxorubicin (μ g) [nmol]	12, [20.7]	12, [20.7]	12, [20.7]	12, [20.7]	12, [20.7]	12, [20.7]
Measured amount from fluorescence	0.479	12.667	0.562	12.306	0.535	12.150
Percent intercalated	96.0 $\pm$ 0.1	-	95.3 $\pm$ 0.5	-	95.5 $\pm$ 0.1	-
Solvent [1000 μ L added to 200 μ L aliquot = 2 μ g]	Acidified Methanol (1% acetic acid)		Acidified Acetonitrile (0.1% formic acid)		Isopropanol	
Recovered in Solvent ^a μ g [%]	0.3 $\pm$ 0.1 [15.1]	2.2 [>100]	0.7 $\pm$ 0.1 [34.7]	2.9 [>100]	0.2 $\pm$ 0.1 [11.2]	2.2 [>100]
Recovered in Digest ^b μ g [%]	1.82 $\pm$ 0.1 [91.5]	0.03 [<2]	1.59 $\pm$ 0.2 [79.7]	0.04 [<2]	1.71 $\pm$ 0.2 [85.7]	0.05 [<2]

^aAfter addition of the solvent, the samples were vortex-mixed for 30 seconds. The acidified methanol samples were then left at -80°C for 1 hr prior to centrifugation (15,000g for 5 min), while the acidified acetonitrile and isopropanol samples were centrifuged immediately. The supernatants were transferred to clean tubes for fluorescence measurement (excitation 509 nm, emission 552 nm). ^bThe DNA pellets were dried under vacuum then digested with 150 Kunitz units of DNase I (Millipore-Sigma) in 210 μ L of 8 mM Tris-HCl buffer pH 7.9 containing 2 mM NaCl, 1.2 mM MgCl₂, and 0.2 mM CaCl₂. After a 30-minute incubation at 37°C, 1000 μ L of acidified methanol was added to each sample, which was then vortexed and centrifuged as before, and the supernatants were transferred to clean tubes for fluorescence measurement.

The extraction efficiency of isopropanol acidified by HCl was tested in a further experiment. Two concentrations of HCl were used: 0.75N HCl in isopropanol [11] and 0.075N HCl in isopropanol/water [9:1] [15]. After the addition of the solvents, the samples were mixed and left at -20°C for 20 hrs before centrifugation. The DNA pellets were washed with isopropanol, and the supernatant fractions were combined, and their doxorubicin concentrations were measured spectrofluorometrically as previously. The Pellets were digested with DNase I (30 min at 37°C), and the digests were diluted with isopropanol, and their doxorubicin concentrations were measured spectrofluorometrically. Total doxorubicin recovery with 0.75N HCl was 79.0%, with 71% in the solvent extract and 8% in the digest. Total doxorubicin recovery with 0.075N HCl was 103.0%, with 81% in the solvent extract and 22% in the digest. This suggests that intercalated doxorubicin can be released from DNA by acid hydrolysis, but that too extreme acidic conditions may decrease overall recovery.

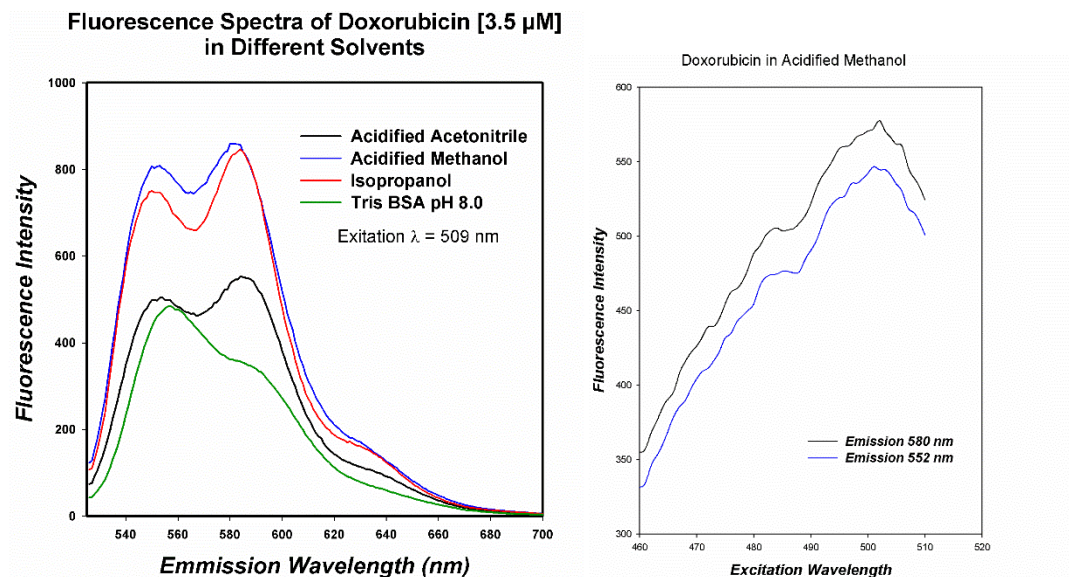


Figure S-7. Emission and excitation spectra of doxorubicin in different solvents (Measured on a Shimadzu RF-5301pc spectrofluorometer).

References:

1. Cshai E, Kangarlou S, Xiang T-X, Ponta A, Bummer P, Choi D, et al. Determination of Key Parameters for a Mechanism-Based Model to Predict Doxorubicin Release from Actively Loaded Liposomes. *Journal of Pharmaceutical Sciences*. 2015;104 3:1087-98; <https://doi.org/10.1002/jps.24307>.
2. Fugit KD, Xiang T-X, Choi DH, Kangarlou S, Cshai E, Bummer PM, et al. Mechanistic model and analysis of doxorubicin release from liposomal formulations. *Journal of Controlled Release*. 2015;217:82-91; <https://doi.org/10.1016/j.jconrel.2015.08.024>.
3. Groman EV, Reinhardt CP. Method to quantify tail vein injection technique in small animals. *ContempTopLab Anim Sci*. 2004;43 1:35-8. <https://pubmed.ncbi.nlm.nih.gov/14984288/>.
4. Riches AC, Sharp JG, Thomas DB, Smith SV. Blood volume determination in the mouse. *JPhysiol*. 1973;228 2:279-84; <https://doi.org/10.1113/jphysiol.1973.sp010086>.
5. Estabrook RW, Werringloer J. The measurement of difference spectra: application to the cytochromes of microsomes. *Methods in Enzymology*. 1978;52:212-20; [https://doi.org/10.1016/s0076-6879\(78\)52024-7](https://doi.org/10.1016/s0076-6879(78)52024-7).
6. Wilhelm S, Tavares A, Dai Q, Ohta S, Audet J, Dvorak H, et al. Analysis of nanoparticle delivery to tumours. *Nature Review Materials*. 2016;1 16014; <https://www.nature.com/articles/natrevmats201614>.
7. Morelli AE, Larregina AT, Shufesky WJ, Zahorchak AF, Logar AJ, Papworth GD, et al. Internalization of circulating apoptotic cells by splenic marginal zone dendritic cells: dependence on complement receptors and effect on cytokine production. *Blood*. 2003;101 2:611-20; <https://doi.org/10.1182/blood-2002-06-1769>.
8. Andersen A, Warren DJ, Slørdal L. Quantitation of cell-associated doxorubicin by high-performance liquid chromatography after enzymatic dequiescence. *Cancer Chemotherapy and Pharmacology*. 1994;34 3:197-202; <https://doi.org/10.1007/bf00685077>.

9. Lucas AT, O'Neal SK, Santos CM, White TF, Zamboni WC. A sensitive high performance liquid chromatography assay for the quantification of doxorubicin associated with DNA in tumor and tissues. *J Pharm Biomed Anal.* 2016;119:122-9; <https://doi.org/10.1016/j.jpba.2015.11.035>.
10. Walters K, Stornetta A, Jacobs F, Villalta PW, Razzoli M, Grant M, et al. Identification of new candidate biomarkers to support doxorubicin treatments in canine cancer patients. *BMC Vet Res.* 2021;17 1:378; <https://doi.org/10.1186/s12917-021-03062-x>.
11. Laginha KM, Verwoert S, Charrois GJ, Allen TM. Determination of doxorubicin levels in whole tumor and tumor nuclei in murine breast cancer tumors. *Clin Cancer Res.* 2005;11 19 Pt 1:6944-9; <https://doi.org/10.1158/1078-0432.ccr-05-0343>.
12. Song G, Darr DB, Santos CM, Ross M, Valdivia A, Jordan JL, et al. Effects of tumor microenvironment heterogeneity on nanoparticle disposition and efficacy in breast cancer tumor models. *Clin Cancer Res.* 2014;20 23:6083-95; <https://doi.org/10.1158/1078-0432.ccr-14-0493>.
13. Dadpour S, Mehrabian A, Arabsalmani M, Mirhadi E, Askarizadeh A, Mashreghi M, et al. The role of size in PEGylated liposomal doxorubicin biodistribution and anti-tumour activity. *IET Nanobiotechnol.* 2022;16 7-8:259-72; <https://doi.org/10.1049/nbt2.12094>.
14. Mazzucchelli S, Ravelli A, Gigli F, Minoli M, Corsi F, Ciuffreda P, et al. LC-MS/MS method development for quantification of doxorubicin and its metabolite 13-hydroxy doxorubicin in mice biological matrices: Application to a pharmaco-delivery study. *Biomed Chromatogr.* 2017;31 4; <https://doi.org/10.1002/bmc.3863>.
15. Gabizon AA. Selective tumor localization and improved therapeutic index of anthracyclines encapsulated in long-circulating liposomes. *Cancer Res.* 1992;52 4:891-6; <https://pubmed.ncbi.nlm.nih.gov/1737351/>
16. Jin Z, Xiao X, Gui L, Lu Q, Zhang J. Determination of doxorubicin in plasma and tissues of mice by UPLC-MS/MS and its application to pharmacokinetic study. *Heliyon.* 2024;10 15:e35123; <https://doi.org/10.1016/j.heliyon.2024.e35123>.
17. Gabizon AA, Pappo O, Goren D, Chemla M, Tzemach D, Horowitz AT. Preclinical Studies with Doxorubicin Encapsulated in Polyethyleneglycol-Coated Liposomes. *Journal of Liposome Research.* 1993;3 3:517-28; <https://doi.org/10.3109/08982109309150734>.
18. Kiyomiya K, Matsuo S, Kurebe M. Mechanism of specific nuclear transport of adriamycin: the mode of nuclear translocation of adriamycin-proteasome complex. *Cancer Res.* 2001;61 6:2467-71; <https://pubmed.ncbi.nlm.nih.gov/11289116/>.